ELECTRICAL CHARACTERISTICS OF AN IDENTIFIED INSECT MOTONEURONE CELL BODY : A CURRENT- AND VOLTAGE-CLAMP STUDY

Wendy Denise Nightingale

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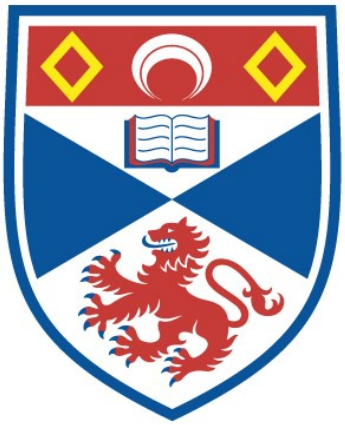

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ELECTRICAL CHARACTERISTICS OF AN IDENTIFIED INSECT MOTONEURONE CELL BODY: A CURRENT- AND VOLTAGE-CLAMP STUDY.

by

Wendy Denise Nightingale.

University of St Andrews.

Department of Biology and Preclinical Medicine.

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.... insects have
their own point
of view about
civilization a man
thinks he amounts
to a great deal
but to a
flea or a
mosquito a
human being is
merely something
good to eat ....

Archy & Mehitabel
By Don Marquis
(FABER).

I, Wendy Nightingale, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date

23:9:1988

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 and as a candidate for the degree of Ph.D. on October 1st. 1984.

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

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So many people have contributed in various ways to my work that I find it hard to name everyone concerned. So finally, many thanks to everyone working at the Gatty Marine Laboratory, past and present.

- CONTENTS -

SUMMARY.

1. The electrical characteristics of the cell body of an identified excitatory motoneurone (cell 3) from the cockroach (*Periplaneta americana*) have been studied under current- and voltage-clamp.

2. Under voltage-clamp depolarising command pulses evoked an outward current which increased with the magnitude of the command step up to approximately +100mV, a component of the current developed more slowly and took longer to reach a maximum. With increasing depolarisation the outward current response fell to a lower level before further increasing. This current response gave rise to a characteristic N-shape I-V relationship. The position of the negative conductance region depends on the time current measurements are taken after the onset of the command pulse.

3. Externally applied cadmium (1mM) or manganese ions (5mM) abolished the slowly developing current responsible for the hump in the I-V relationship. These results indicate that calcium ions are required for the activation of this component of the outward current. Verapamil (50μM) also reduced this current component and appeared to be non-specific in reducing another current component. Furthermore, verapamil caused inactivation of the remaining current which was more marked for long duration (500ms) command pulses.

4. Externally applied TEA^+ (at concentrations greater than 25mM) blocked the calcium-dependent current and a calcium-independent component. Under current-clamp TEA^+ (50mM) unmasked a broad action potential.

5. Externally applied aminopyridines did not enhance excitability under current-clamp. Under voltage-clamp aminopyridines had significant effect in shifting the voltage dependence of the hump in the I-V relationship toward more negative potentials.

6. When holding at -90mV and stepping to more positive potentials there was no indication of an early, fast, transient component similar to I_A. If present at all, I_A made only a minor contribution to the total outward currents.

7. A double command pulse regime was used to study tail currents whereby a standard pre-pulse (pulse (I)) was immediately followed by a test pulse (pulse (II)) to various command potentials. Tail current measurements were taken during pulse (II). The tail currents showed strong outward rectification and were severely reduced in saline containing cadmium ions (1mM).

8. The tail-current reversal potential was dependent on the pulse (I) magnitude and duration. Preliminary results indicated that increasing the pulse (I) magnitude caused a negative shift in reversal potential. Increasing the pulse (I) duration from 10ms to 50ms caused a positive shift in the reversal potential equivalent to a two-fold increase in extracellular cation concentration.

9. A five-fold increase (from 3.1 to 15mM) in external potassium ion concentration produced a small and variable shift in reversal potential, which did not conform to that predicted by the Nernst equation. A five-fold decrease (from 235 to 47mM) in external chloride ion concentration had little effect on the tail current reversal potential but did cause a slight reduction in the outward

currents. Furthermore, the voltage dependency of the hump in the I-V relationship was shifted toward more negative potentials.

10. Action potentials induced by intracellular citrate injection were only slightly enhanced by a four-fold increase (from 9 to 36mM) in external calcium ion concentration. They were reversibly reduced to a graded spike in saline containing verapamil (10μM) and reversibly abolished by manganese ions (40mM), but were relatively unaffected by sodium-free saline. These observations suggest that calcium ions were the major ion carrying the inward current under these conditions.

11. Carbon dioxide-induced action potentials were reversibly reduced to a graded spike in sodium-free or manganese saline (40mM) whereas tetrodotoxin (50nM) irreversibly abolished action potentials for wash period up to 20mins. These observations suggest that both calcium and sodium ions were responsible for the inward current under these conditions.

12. The regenerative component of the axotomy-induced action-potentials was reversibly reduced in sodium-free saline and only partially reduced with some broadening in calcium-free or manganese saline (40mM). Either treatment alone was insufficient to completely abolish or reduce the action potential to a graded spike. A combination of Na-free saline with manganese ions (40mM) caused a more complete block by reducing the regenerative component to a graded spike. These results suggest that sodium ions, and to a lesser extent, calcium ions were responsible for the inward current under these conditions.

1. INTRODUCTION.

1.1 HISTORICAL BACKGROUND

1.11 Introduction.

At the beginning of the 20th century a number of thermodynamic and electrostatic laws were proposed in order to describe the properties of electrolytes in solution. These laws were wholely theoretical and could be equally applied to biological and non biological systems, though they were of particular relevance to the properties of excitable cells. At that time they were not supported by experimental biological data. They still form the basis, however, of present day understanding of impulse generation.

1.12 Permeability and Conductance changes.

Physiochemical studies by Nernst revealed large electrical potentials arising from the diffusion of electrolytes down their electrochemical gradients across a semi-permeable membrane. When the net movement of electrolytes stops the system has an equilibrium potential. At the turn of the century Julius Bernstein (1902) formulated the "membrane hypothesis" whereby excitable cells possess a selectively permeable outer membrane which has a positive charge on the outside and a negative charge on the inside. At rest this membrane was considered selectively permeable to potassium. During excitation Bernstein proposed that this selectivity is lost, enabling other ions to cross the membrane thereby reducing the internal negativity and causing the membrane potential to shift toward zero. This was known as "membrane breakdown".

Cole and Curtis (1938; 1939) began investigating membrane properties of the alga *Nitella* and the squid axon using a high frequency Wheatstone bridge circuit. Cells were placed between two extracellular electrodes and their impedances measured during

activity. They discovered that each action potential from these vastly different cells was accompanied by a dramatic impedance decrease. In the squid axon this was largely due to a 40-fold increase in membrane conductance with very little change in capacitance. Bernstein's proposal of a membrane permeability increase was essentially supported by Cole and Curtis.

Although Bernstein's membrane hypothesis and application of Nernst's treatment of electrochemical gradient are based on the view that the resting membrane is relatively impermeable to all ions except potassium, this is an oversimplification. Sodium ions do leak into the cell, albeit relatively slowly. Sodium influx will slightly depolarise the membrane which in turn will place chloride ions out of equilibrium and they will cross the membrane. The situation was considered by Goldman in 1943, and he derived an equation to relate membrane potential to ionic concentration ratios and their relative permeability coefficients.

$$V_m = 58 \log \frac{K_o + (P_{Na}/P_K)\, Na_o + (P_{Cl}/P_K)\, Cl_i}{K_i + (P_{Na}/P_K)\, Na_i + (P_{Cl}/P_K)\, Cl_o} \qquad \text{Eq. 1.12 1}$$

Where P_{Na}, P_K and P_{Cl} are defined as:

$$P_{ion} = \frac{uBRT}{aF} \qquad \text{Eq. 1.12 2}$$

Where u is the mobility of an ion in the membrane, B is the partition coefficient between membrane and aqueous solution, a is the thickness of the membrane, R is the gas constant, T is the absolute temperature and F the Faraday constant. In the squid axon the relative permeabilities are:

K	:	Na	:	Cl
1.0		0.03		0.1

The equation is derived assuming that the individual currents are independent of one another, and that the ions traverse the membrane by simple diffusion down a potential gradient. This is also known as

"the constant field theory" because it is also assumed that the electrical potential gradient is uniform across the membrane. Permeability is a measure of leakiness of the cell membrane to a particular ion and is independent of ionic concentration. Despite the resting permeability of the membrane, the internal concentration of ions remains fairly constant. This is achieved by an active transport process requiring metabolic energy to exchange external potassium for internal sodium ions. This ionic pumping is achieved through the membrane Na-K ATPase or sodium pump (for a review see Dahl and Hokin, 1974)

Ionic currents are related to membrane potential by their conductances. Conductance, termed g (reciprocal of resistance), is a measure of the ease with which charge will flow and is thus an indication of membrane permeability.

Consider Ohms' Law: $I = \frac{V}{R}$ Eq. 1.12 3

Where I is the current (amps), V is the voltage drop (volts) and R is the resistance (Ohms).

Substituting g:

$$I = gV \qquad \text{Eq. 1.12 4}$$

$$I_{ion} = g_{ion} (V_m - E_{ion}) \qquad \text{Eq. 1.12 5}$$

Where $(V_m - E_{ion})$ is the difference between the membrane potential (V_m) and the equilibrium potential for a given ion (E_{ion}) which equals the driving force on that ion. When $V_m = E_{ion}$, the driving force is zero and the net current flow is zero regardless of membrane permeability or conductance. The conductance, however, is related to the permeability and is also known as "the constant of proportionality" relating current to the membrane potential. This is also known as the chord conductance.

1.13 Recording the action potential.

A breakthrough in the understanding of impulse generation came when, for the first time, Hodgkin and Huxley (1939; 1945) and Curtis and Cole (1940; 1942) measured action potentials in squid axons using intracellular electrodes. At the peak of the action potential the intracellular charge did not disappear as was originally assumed. Instead the transmembrane potential overshot 0mV and became reversed. This overshoot was attributed by Hodgkin and Katz (1949) to a switch in the selective permeability of the axon membrane to external Na^+ which entered the cell during the action potential and took the internal electrical potential of the cell toward the equilibrium potential for sodium (+60mV). They tested their hypothesis by reducing the external sodium ion concentration, replacing it with sucrose, glucose or choline. In low sodium solutions the action potential rose more slowly and the amplitude was smaller. These results confirmed their sodium hypothesis. These experiments marked the unfolding of the ionic basis of membrane excitability.

1.14 Transmembrane Current Quantification.

Up to the late 1940's the passage of ions across a membrane and the resultant current flow had been qualitatively described in terms of selective sodium and potassium conductances. In order to quantitatively assess electrical activity there were some experimental considerations. The action potential has a curious shape as a result of the voltage and transmembrane currents changing as a function of time. In addition the membrane acts as a capacitor and a small proportion of the current flowing will be due to the discharging of the membrane capacitance as the membrane potential changes. With the three interdependent variables, current, voltage and time, quantitative assessment was not feasible.

Analysis of transmembrane currents became possible with the development of a revolutionary type of experimental procedure in the late 1940's called voltage-clamp. The technique was devised independently by Marmot (1949) and Cole (1949), and further developed by Hodgkin, Huxley and Katz (1952). The voltage-clamp technique involved controlling the voltage across a cell membrane by a negative feedback circuit and directly measuring the resultant ionic movements as small transmembrane currents. In such experiments two intracellular electrodes were used. The first electrode measured the membrane potential and was connected to the "inverting" (negative) input of a differential feedback amplifier. The non-inverting (positive) input was connected to a variable voltage source and could be controlled by the experimenter (Fig. 1.14 1). The output, therefore, was amplified and opposite to the signal discrepancy and the system acted as a negative feedback loop. The differential amplifier delivered current when there was a voltage discrepancy between its two inputs therby clamping the membrane at the desired voltage. The potential at which the cell was held was called the holding potential (V_H). The cell membrane potential could be stepped positive or negative from this value to a new potential called the command potential (V_C). When the membrane potential was stepped to a new level, the current flowing was a measure of the transmembrane ionic and capacitative currents which were monitored by a bath electrode connected to a current monitor. The feedback amplifier needed to have a high frequency response in order to rapidly counteract any potential fluctuation. In addition, the compliance of the equipment needed to be high in order to inject sufficient current during depolarisations which would otherwise be accompanied by large potential fluctuations. Another important consideration was that the area of membrane under investigation must be isopotential, the so

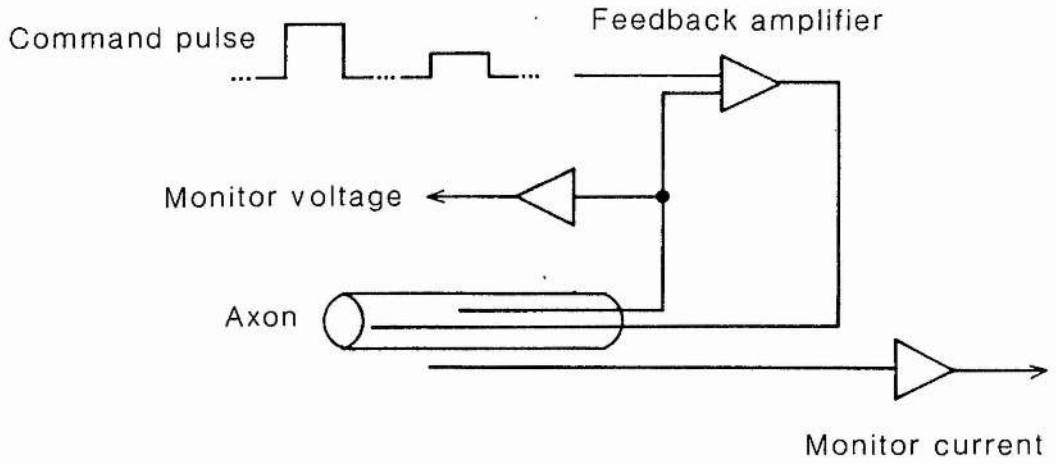


Fig. 1.14 1

Schematic diagram of a voltage-clamp circuit. The axonal membrane potential was monitored by a longitudinal intracellular electrode. The output was then fed into a differential feedback amplifier together with the imposed experimental command pulse. When the membrane potential differed from the command pulse the amplifier produced an amplified and inverted current signal which was fed into the cell via the second intracellular electrode in order to annul the potential change.

called space-clamp condition. In the squid axon this was achieved by inserting a fine silver wire longitudinally along virtually the whole length of the giant axon preparation.

In the squid giant axon the membrane potential was held near its physiological resting potential and sequentially stepped to more positive potentials. By varying the external Na^+ concentration these classical experiments identified two separate ionic currents in the form of voltage-dependent Na^+ and K^+ currents superimposed upon a minor leakage current of undetermined ionic origin (Hodgkin and Huxley, 1952a). Hodgkin and Huxley then proceeded to quantitatively describe the ionic permeabilities by measuring the "instantaneous current-voltage relation" (Hodgkin and Huxley, 1952b). Under voltage-clamp the axon was first depolarised sufficiently to raise the permeability and then stepped to a new command potential. The current was measured 10-30μs after the second step before further permeability changes had taken place. When Na and K permeabilities were high the I-V relationship was linear according to Ohms law (see Eq. 1.12 5).

The Hodgkin-Huxley model (1952c) proposed that ionic conductances describe permeability changes brought about by the opening and closing kinetics of ionic channels. These channels are responsible for the rising and falling phase of the action potential. Activation is the rapid process that opens Na channels during a voltage-clamp step depolarisation, and allows gNa to increase with a very steep voltage dependency toward a maximum value. Beyond this maximum value gNa shows a slower time- and voltage-dependent decline as the Na channels close during a depolarising step, a process known as inactivation. Once the Na channels have been inactivated they cannot be activated to the conducting state until their inactivation

has been removed by repolarising or hyperpolarising the membrane. While Na inactivation is taking place, K channel activation occurs much more slowly and achieves a new steady state during the depolarisation. Squid potassium channels do not show inactivation during short duration depolarisations. Upon repolarisation the gK slowly returns to the resting level.

Hodgkin and Huxley (1952c) then developed a mathematical description for the time-course of the Na and K conductances during depolarising voltage steps. The voltage and time-course relationship for g_K follows an S-shaped time course followed by an exponential decline on repolarisation. For potassium this could be described as a first order kinetics exponential (n) raised to the fourth power:

$$g_K = g_{Kmax}\, n^4 \qquad \text{Eq. 1.14 1}$$

Where g_{Kmax} is the maximum conductance for a given voltage step and n^4 is the probability of four particles being in the correct position for the channel to open during depolarisation. A first order process involves only one type of molecule as a reactant. In this equation the molecule type is n particles which make transitions between permissive and nonpermissive forms i.e. activated and inactvated states. Note that there is no inactivation term for potassium in this system.

Sodium also exhibits an S-shaped conductance curve and its overall time course consists of an activation and an inactivation term. The activation was fitted by a first order kinetics exponential (m) raised to the third power, presumably because three particles associated with the Na channel must be in the correct position for it to open. Inactivation was a straight forward first-order decay (h):

$$g_{Na} = g_{Namax}\, m^3 h \qquad \text{Eq. 1.14 2}$$

From these two equations (Eq. 1.14 1 & 2) Hodgkin and Huxley were

able to reconstruct the action potential as a function of time and changing conductances. Their theoretical values closely matched the actual action potential recorded from a squid axon.

Subsequent investigators have shown that the fundamental principles of voltage-dependent permeability changes and concomitant conductance changes brought about by opening and closing of Na and K channels are generally applicable to other excitable membranes.

The mid-1970s saw another major technical development in electrophysiology, namely the patch-clamp technique (Neher and Sakmann, 1976; Hamill, Marty, Neher, Sakmann and Sigworth, 1981). This technique made it possible to resolve microscopic currents that correspond to the opening and closing of individual ion channels. In practice, a polished glass pipette (tip diameter 0.5-1.0μM) is placed on the cell surface of enzymatically cleaned cells, and then gentle suction is applied in order to achieve a seal between the glass and membrane with an electrical resistance in the order of 10-100 gigohms. Starting from the cell-attached mode where the patch pipette is attached to the cell via a high resistance seal, any one of three other recording configurations can be achieved (Fig. 1.14 2), each of which can be used to address different problems (Sakmann and Neher, 1984).

Although patch-clamping has only been developed within the past 12 years, it is now used in many laboratories around the world and on many different types of cell and subcellular organelle in addition to nerve cells (Hille, 1984).

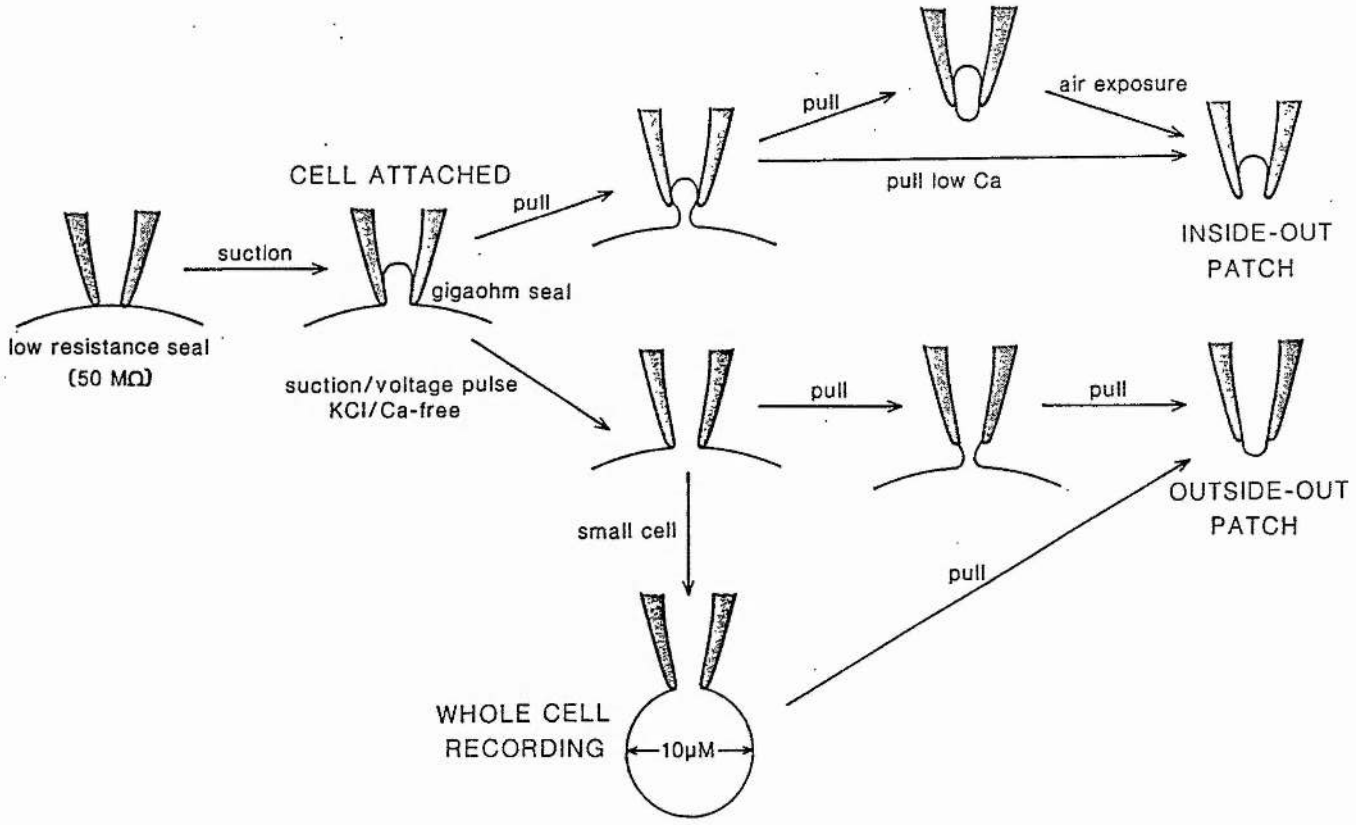


Fig. 1.14 2

A schematic flow diagram of the four patch-clamp configurations and the methodology involved (adapted from Hamil, Marty, Neher and Sakmann, 1981).

1.2 MECHANISMS OF EXCITABILITY.

1.21 Introduction.

The most studied and understood channels in terms of kinetics and, latterly, molecular structure are the Na and K channels of axons. Classically Na^+ ions carry the inward current while K^+ ions carry the outward current. Not long after the sodium theory for the action potential was proposed, Fatt and Ginsborg (1958) identified calcium as the major inward current carrying ion in crab leg muscle fibres. Since this early discovery with crustacean muscle, and indeed with other types of muscle, calcium spikes have been identified in a number of vertebrate and invertebrate preparations. For example, molluscan nerve cell bodies (Oomura, Ozaki and Maeno, 1961), frog spinal ganglion cells (Koketsu, Cerf and Nishi, 1959), *Paramecium* (Naitoh, Eckert and Friedman, 1972), starfish eggs (Hagiwara, Ozawa and Sand, 1975), mammalian hippocampal cells (Dingledine, 1983) and guinea-pig olfactory cortex neurones (Galvan, Constanti and Franz, 1985). The excitability of a cell is a balance between ions which generate inward current e.g. Na^+ and Ca^{2+}, and those which generate outward current i.e. those which tend to depolarise and those which tend to hyperpolarise the cell e.g. K^+ and Cl^-. Using old terminology, potassium channels serve to "stabilise" the membrane, i.e. to maintain the membrane potential near the potassium equilibrium potential and away from threshold. No other channels offer the diversity shown by potassium-selective channels which can be activated by voltage, ions, transmitters, and intracellular messengers. By combining different channel types subtle physiologically functional requirements can be achieved.

1.22 POTASSIUM CURRENTS.

1.221 Delayed Rectifier (I_K).

In the classical papers of Hodgkin and Huxley (1952a; b; c), the K^+-selective channel in axonal membranes was called the "delayed rectifier", so called because of the delay in its activation after a voltage step depolarisation and rectifier because current flows more readily in one direction, out of the cell. Under voltage-clamp conditions squid axonal I_K slowly activates around -30mV and was originally reported to remain at a steady state (Hodgkin and Huxley, 1952c). Later work by Ehrenstein and Gilbert (1966) showed that with prolonged depolarisation (4-5 seconds) I_K rises to a peak then undergoes slow inactivation to a steady state value. This inactivation of I_K also occurs in molluscan neurones (Connor and Stevens, 1971a; Thompson, 1977; Aldrich, Getting and Thompson, 1979).

In molluscan neurones there is a progressive decline in outward current upon repetitive stimulation (<2Hz) caused by continued inactivation during the inter-pulse interval. This phenomenon has been termed cumulative inactivation and can affect the shape of the action potential i.e. cause broadening (Aldrich *et al.*, 1979). Inactivation of I_K is voltage as well as time-dependent, but does not require Ca^{2+} influx. When measured by the effect of pre-pulses of varying duration on the peak amplitude of a test-pulse current, the time course of inactivation is best fitted by the sum of two exponential functions with time constants of 570ms, and 3.5ms. The rapid inactivation component accounts for 90% inactivation and has a time course comparable to its activation (Aldrich *et al.*, 1979).

Recent work on internally dialysed squid axon has indicated the involvement of ATP in the modulation of the gating mechanism of I_K channels (Benzanilla, Caputo, DiPolo and Rojas, 1986). The gating mechanism of a channel refers to a "sensor" within the channel which

opens or closes the channel in response to appropriate stimuli. When ATP is added to the saline, the potassium current, for pulses more positive than -30mV, is increased by 2.5-fold. Removing ATP altogether reduces the peak outward current. Conversely the current for pulses more negative than -30mV is reduced. ATP decreases the activation and hastens the inactivation when Mg^{2+} ions are present in the dialysis media. The pyrophosphate groups of ATP have a high affinity for divalent cations, thus active ATP is found as a 1:1 metal complex with magnesium in the cytoplasm. Magnesium ions were therefore required within the perfusate for ATP activity. Benzanilla *et al.* (1986) suggest a specific phosphorylating site of the potassium channel which subsequently alters the voltage sensor of the activation/inactivation gating thereby shifting the voltage dependence to more positive membrane potentials.

I_K from different preparations eg. frog node of Ranvier (Armstrong and Hille, 1972), squid axon (Tasaki and Hagiwara, 1957; Armstrong, 1969) and molluscan neurons (Neher and Lux, 1972; Thompson, 1977; Hermann and Gorman, 1981b) is blocked by tetraethylammonium ions (TEA^+ see APPENDIX IIIB for structure) applied from the inside or outside of the membrane. TEA^+ has differential effects upon potassium currents depending on its concentration and whether it is applied internally or externally to the membrane (for review see Stanfield, 1983). Only internally applied TEA^+ was effective in blocking I_K in squid giant axons (Tasaki and Hagiwara, 1957). Conversely in molluscan neurones externally applied TEA^+ greatly reduced I_K leaving the inward current unaffected (Connor and Stevens; 1971a) whereas internally applied TEA^+ blocks the fast transient potassium current (I_A) and I_K equally (Neher and Lux, 1972).

These observations can be explained if there are two receptors for TEA^+, one accessed from the cytoplasm and the other from outside the cell. According to Armstrong's theory for quarternary ammonium ion (QA) block (reviewed by Armstrong, 1971) the QA receptor lies within the channel pore so that blockade can only occur when the channel is opened by a depolarising pulse allowing QA to enter from the internal surface of the cell membrane. The channel can also close with QA in place. The quarternary ammonium series includes: TEA^+, nonyltriethylammonium (C9), tetrapentylammonium (TPeA), and other hydrophobic derivatives. At potentials which cause inward K^+ currents, external K^+ speeds the rate of drug dissociation by physically ousting the QA from the open channel. This internal receptor is distinct from the external one which is rapidly blocked at higher concentrations of QA and is relatively unaffected by membrane potential, gating, or external K^+ (Hille, 1984). The effect of TEA^+ on I_{KCa} wil be reviewed in section 1.223.

Other drugs and toxins have been found to affect I_K in different preparations. For example, external application of the drug quinidine to molluscan pacemaker neurones affects I_A, I_K, and the calcium-activated potassium current (I_{KCa}) (Hermann and Gorman, 1984). The primary action of this drug is the rapid suppression of I_K in a manner similar to QA's effect (Armstrong, 1971) at low concentrations (100μM) in a voltage-dependent manner. Phalloidin is a cyclic heptapeptide isolated from the toadstool *Amanita phalloides*. At low concentrations (10^{-14} to 10^{-8}M) under current-clamp conditions, the toxin significantly increased the action potential duration and the amplitude of the associated contraction in intact frog skeletal muscle (Cognard, Ewané-Nyambi, Potreau, and Raymond, 1985). Voltage-clamp studies showed that phalloidin reversibly decreased I_K. Further investigations indicated that the mechanism of

inhibition was voltage-dependent and frequency-independent. More positive holding potentials facilitated toxin-blockade (Cognard, Ewané-Nyambi, Potreau, and Raymond, 1986).

Noxiustoxin (NTX) is isolated from the venom of the scorpion *Centruroides noxius*. Carbone *et al.* (1987) reported that application of NTX (5μM) to isolated squid giant axons causes almost full blockade of I_K at 0mV command potential, but at 100mV I_K was only 60% reduced. The kinetics and amplitude of the Na^+ current was little affected by the toxin. The blocking action appears to be voltage-dependent. At toxin concentrations of less than 1.5uM, however, the blocking action is voltage-independent (Carbone, Wanke, Prestipino, Possani and Maelicke, 1982), which also suggests a concentration dependency. In addition , the toxin blocking potency at concentrations less than 1.5μM, was modified by holding potential; a greater block was observed at more negative potentials. Repetitive pulses and strong depolarisations relieved the channel block in a manner similar to that reported for 4-aminopyridine (4-AP) in squid axons (Meves and Pichon, 1977). Total blockade of I_K by NTX was not achieved.

There is also a toxin called toxin II-9 (Possani, Martin and Svendsen, 1982) isolated from the Latin American scorpion *Tityus serrulatus*. This toxin has similar physical and physiological properties to NTX (Carbone, Prestipino, Spadavecchia, Franciolini and Possani, 1987).

In summary, the physiological significance of I_K would be membrane repolarisation after an action potential ensuring that the action potential is brief. The phenomenon of cumulative inactivation can have an effect upon the shape of the action potential i.e. cause broadening (Aldrich, Getting and Thompson, 1979). The delayed

rectifier (I_K) channel is most commonly found in axonal membranes and also occurs in neuronal cell bodies along with other types of K channels. I_K slowly activates at potentials more positive than -30mV and undergoes slow inactivation. External application of TEA^+ preferentially blocks I_K at low concentrations. This action, however, is not entirely specific and can depress I_{KCa} in some preparations. Internal application of TEA^+ blocks other K channels equally well. Quinidine also non-specifically blocks I_K. A number of toxins are relatively specific for I_K e.g. phalloidin, the venom extracts NTX, and toxin II-9.

1.222 Early, Fast Transient (I_A).

Many excitable cells possess a distinct population of potassium channels that rapidly activate and inactivate producing a transient outward current. This current was originally reported by Hagiwara, Kusano, and Saito (1961) in molluscan (*Onchidium*) neurones. A decade later Connor and Stevens (1971b) and Neher (1971) further characterised the current in molluscan cells and termed it I_A (or the A-current). I_A has been reported in invertebrates, including molluscan neurones, insect muscle and starfish oocyte, and vertebrates, including mammalian, fish and amphibian neurones, mammalian muscle and endocrine cells (for a review see Rogawski, 1985).

Stepping the membrane potential from a hyperpolarised potential (-100mV) back to the region of the resting potential (between -50 and -35mV) results in a transient outward potassium current in some neurones (Connor and Stevens, 1971b; Neher 1971; Thompson, 1977). I_A exhibits rapid activation (10-20 ms) and an exponential inactivation (200-600ms) during steady-state depolarisation. The time course of I_A, although much slower, resembles voltage-dependent sodium channels described by the first order equations of Hodgkin and Huxley (1952c). Inactivation can only be removed by a conditioning hyperpolarisation. Single channel conductance measurements of I_A have been made in a number of preparations. Taylor (1987) reported a value of 14pS in *Helix aspersa* neurones, Cooper and Shrier (1985) reported a conductance of 22pS in rat nodose ganglia cells, whilst the largest conductance of 40pS was reported in *Lymnaea stagnalis* neurones (Kazachenko and Geletyuk, 1984). The kinetics of I_A enabled it to be separated from the extensively studied I_K. A recent study by Taylor (1987) demonstrated that I_A channels of *Helix aspersa* neurones are permeable to a number of mono-valent cations. The

permeability sequence being $Tl^+ > K^+ > Rb^+ > NH_4^+$.

The pharmacological profile of I_A is also distinct from I_K. Thompson (1977) reported that I_A was selectively blocked by low concentrations (3 mM) of externally applied 4-AP and was much less sensitive to external TEA^+ or to Co^{2+} and Mn^{2+} than I_K. At high concentrations or with internal application, however, 4-AP does affect other potassium currents (Hermann and Gorman, 1981a). Throughout several phyla, I_A is blocked by aminopyridines with a similar dose dependency. This shows pharmacological similarity and implies conservation of the channels' molecular structure (Rogawski, 1985).

Using cockroach giant axons, Pelhate and Sattelle (1982) proposed a rank potency for aminopyridines and their derivatives. The most critical molecular features seem to be: lipid solubility; a positively charged amino group; and small molecular size. Hence the smaller molecules such as 3,4-diaminopyridine (3,4-DAP) and 4-AP were more selective for K^+ channels whereas 4-aminoquinoline (4-AQ) also blocked Na^+ channels. In squid the aminopyridine blockade appears to be dependent on membrane potential and frequency of stimulation. Blockade is relieved by repetitive depolarising pulses and restored by prolonged repolarisation due to the molecules being displaced from their binding sites (presumably by potassium ions) and slowly rebinding (Meves and Pichon, 1977).

Although Thompson (1977) reported a 40% reduction in I_A in molluscan neurones after external application of Co^{2+}, this was ascribed to the indirect surface charge effects causing a shift in steady-state activation/inactivation toward the positive region of the voltage axis of the I-V curve i.e. a similar shift would occur with elevated external Ca^{2+}. More recently, however, Junge (1985) has reported that I_A in *Aplysia californica* neurones was blocked by

external application of Co^{2+} and augmented by increasing external $[Ca^{2+}]$. Further evidence for the involvement of Ca^{2+} was gained by the application of the organic Ca channel blocker, verapamil hydrochloride, which blocked I_A in *Aplysia* neurones (Junge, 1985). The calcium-activated I_A was also blocked by aminopyridines. A calcium-activated I_A has also been reported in the muscle membrane of adult and larval wild-type and mutant *Drosophila* (Salkoff, 1983a).

Low concentrations (5-10μM) of externally applied TEA^+ will selectively reduce I_K, but higher concentrations (20-100mM), however, will also block I_A (Connor and Stevens, 1971b ; Neher and Lux, 1972 ; Thompson, 1977). The different sensitivities were demonstrated by Neher and Lux (1972) by a 5-fold increase in TEA^+ concentration required to block I_A to the same degree as I_K.

Dendrotoxin (DTX) is a polypeptide isolated from the venom of the Green Mamba snake *Dedroaspis angusticeps*. This toxin has been reported to selectively reduce a portion of the delayed non-inactivating outward current (I_K) in guinea-pig dorsal root ganglion neurones leaving the fast inactivating component (I_A) unaffected (Penner, Petersen, Pierau and Dreyer, 1986). External TEA^+ reduced this current and subsequent addition of DTX was able to further reduce a portion of the outward current. In this preparation, however, 3,4-DAP partially reduced I_K and subsequent application of DTX had no effect, presumably because DTX has an aminopyridine-like blocking action. Pharmacologically, this current resembles the fast transient current more than the delayed rectifier. The specificity of DTX appears to vary with the cell type and has been reported to selectively block I_A in hippocampal neurones (Dolly *et al.*, 1984; Halliwell, Othman, Pelchen-Matthews and Dolly, 1986).

The fruit fly *Drosophila melanogaster* has a genetic defect form called a "shaker" mutant, so-called because under ether anaesthesia

their legs shake. Treating normal wild-type animals with 4-AP could mimic the shaker mutant, which suggested an abnormal potassium channel (Jan, Jan and Dennis, 1977). This was confirmed by Salkoff (1983b), who reported that adult shaker mutants have modified myotube I_A channels.

Wild-type adult flies have two types of transient outward currents in muscle membrane. One is I_A and the other is a calcium-dependent fast outward current termed I_{Acd} (Salkoff, 1983a). There is also an additional slowly developing outward current. These two transient currents have a similar time-course and are superimposed on the fast inward current which makes analysis difficult. Advantage can be taken of I_{Acd} being preferentially suppressed by Co^{2+} (10mM), La^{3+} (1mM) and TEA^{+} (20mM), while I_A is unaffected. Likewise 3,4-DAP blocks I_A leaving I_{Acd} unaffected (Gho and Mallart, 1986). The adult shaker mutant, however, has an altered or absent I_A (Salkoff, 1983b), but does have I_{Acd} (Salkoff, 1983a). Studies by Gho and Mallart (1986) have shown that I_{Acd} is also present in wild-type and mutant larval (third instar) muscle fibres while I_A is absent from mutant larvae. The physiological significance of I_{Acd} appears to be the rapid repolarisation after the phasic entry of calcium during an action potential (Salkoff, 1983a).

Cultured myotubes and neurones of *Drosophila* have quite distinct I_A channels called A1 and A2 respectively (Solc, Zagotta and Aldrich, 1987). The type A1 myotube channels have faster and more voltage-dependent macroscopic inactivation kinetics, a larger single channel conductance (between 12-16pS) and inactivate at more positive potentials than the type A2 neuronal channels. Solc *et al.* (1987) reported that the myotube A1 channels are susceptible to Shaker mutations while the neuronal A2 channels are not, which suggests the

involvement of different genes in the expression of I_A in *Drosophila*.

With increasing information being accumulated on the genetic mapping of *Drosophila* genes, future research may lead to deliberately altered channels and their consequential electrical activity being investigated. These experiments would be the basis of molecular structure-activity relationships. Already present investigators have mapped the sequential development of muscle membrane channels between pupal and adult stages. In the pupal stage the first current to appear is I_A followed by I_K, while at the pupal-adult transition I_{KCa} appears shortly followed by I_{ACd} (Salkoff, 1983a; 1985; Salkoff and Wyman, 1981).

The physiological importance of I_A appears to be in enabling the encoding of graded depolarisations into a spike train frequency (Connor and Stevens, 1971b; Hille, 1984; Rogawski, 1985). The sum of both the inhibitory and excitatory graded inputs onto a neurone are transformed into a code of spike trains. In effect I_A increases the interspike interval between action potentials thereby slowing the rate of repetitive firing to a maintained stimulus (Adams, Smith, and Thompson, 1980). Upon repolarisation after an action potential the cell hyperpolarises beyond the resting potential due to strong activation of I_K. Eventually the voltage-dependent I_K channel closes, allowing the cell to depolarise. The I_A channel now re-opens and effectively counteracts the stimulus input thereby increasing the interspike interval until I_A inactivates and depolarisation ensues (Connor, 1978).

In summary, I_A is rapidly activated at potentials between -100 and -30mV producing a transient outward current which rapidly inactivates during a sustained depolarising pulse. The distinct kinetics and pharmacological profile of I_A enable it to be readily separated from I_K. The aminopyridines and their derivatives

selectively depress I_A; 4-AP and 3,4-DAP being the most potent. High concentrations of externally applied TEA^+ will also non-specifically block I_A. In some species I_A is further divided into calcium-independent and calcium-dependent sub-types, where calcium antagonists are able to suppress the latter.

1.223 Calcium-activated (I_{KCa}).

In molluscan neurones the delayed outward current can be pharmacologically divided into two major components: the voltage-dependent, TEA^+-sensitive component called I_K; and a second component, called I_{KCa}, activated by Ca^{2+} entry into the cytoplasm and blocked by external Co^{2+} (Meech and Standen, 1974; Thompson, 1977). A similar current has been identified in a number of different preparations including cat spinal motoneurones (Krnjević and Lisiewicz, 1972; Krnjević, Puil and Werman, 1975), erythrocytes (Lew and Ferreira, 1978), cultured mouse spinal neurones (Lambert, Cottrell, Peters, Green and Newton, 1984), insect motoneurones (Thomas, 1984), vertebrate retinal ganglion cells (Newman, 1985) and plant vacuoles (Hedrich and Neher, 1987).

Intracellular ionophoretic injection of Ca^{2+} into voltage-clamped *Helix* neurones produced a strong outward current (Meech, 1974a; Hermann and Hartung, 1982a; 1982b). Using standard calcium injections (100nA for 10ms) at various holding potentials a non-linear I-V relationship was constructed which gave a reversal potential of -75mV, close to the theoretical reversal potential for a potassium current. This cation dependency results in a characteristic N-shape I-V relationship (Meech and Standen, 1975; Thompson, 1977). With successive clamp depolarisations the curve reaches a maximum at positive membrane voltages then decreases as the Ca^{2+} equilibrium potential is approached resulting in a reduction in the driving force on Ca^{2+} ions and a fall in the net Ca^{2+} influx. Although the total outward current increases once more at more positive potentials this does not represent a further increase in I_{KCa}.

I_{KCa} was thought to exhibit an "apparent" voltage dependency due to the voltage-dependent entry of Ca^{2+}. Meech (1979) proposed that

this was an indirect voltage dependency and the channels could be considered "agonist-gated". However, more recent single channel recordings have shown that I_{KCa} channels have an intrinsic voltage sensitivity (Barrett, Magleby, and Pallotta, 1982). With depolarisation, the rate of channel opening increases and the rate of closing decreases.

At least two types of macroscopic I_{KCa} have been distinguished. The first type is activated by intracellular Ca^{2+}, shows a relatively slow time course and is present in molluscan neurones (Meech, 1974b; Eckert and Tillotson, 1978; Gorman and Thomas, 1978; Hofmeier and Lux, 1981), cat spinal motoneurones (Krnjevic, Puil and Werman, 1975) and neuroblastoma cells (Moolenaar and Spector, 1979). In these preparations I_{KCa} is activated by an elevation in cytosolic free calcium. This calcium may be released from cytosolic stores (Andresen and Brown, 1979) or may reflect a calcium influx from the external solution (Meech, 1974b). The second type is activated by Ca^{2+} influx through the membrane, has a faster time-course (Heyer and Lux, 1976b; Lux and Hofmeier, 1978) and is depressed by an elevated cytosolic Ca^{2+} level (Heyer and Lux, 1976b; Eckert, Tillotson and Brehm, 1981).

In some preparations, Ca^{2+} ions entering the cell have two opposing effects on I_{KCa}. The first is the direct activation of I_{KCa}. The second is an indirect reduction in I_{KCa} via a direct suppression of I_{Ca}. Heyer and Lux (1976b) proposed that Ca^{2+} moving through the membrane is responsible for I_{KCa} activation and also inactivation in *Helix* neurones. Firstly, the time course of I_{KCa} activation and voltage-dependency closely follows that of the inward Ca^{2+} current (termed $I_{in\ slow}$) in *Helix* neurones (Heyer and Lux, 1976a). Secondly, because repetitive depolarising pulses (100ms) at

short repolarisation intervals increased the initial rate of rise, i.e. within 20ms, of the current response of subsequent pulses despite a concomitant reduction in the maximum current amplitude. This augmentation was reversibly abolished in Co^{2+} and Mg^{2+} saline. The shorter the repolarisation interval the greater the augmentation of I_{KCa} because the $I_{in\ slow}$ time constant is sufficiently long that the channel will not have had time to close, $I_{in\ slow}$ is incompletely turned off, and reactivation will be augmented. Furthermore, a hyperpolarising pre-pulse removes the $I_{in\ slow}$ and prevents augmentation even at short repolarisation intervals. Third and finally, holding the cell at more negative potentials e.g. -70mV, reduced the available $I_{in\ slow}$ in between pulses. Under these conditions only the shortest repolarisation time course of 20ms was accompanied by augmentation. From these results the authors concluded that Ca^{2+} moving through the membrane activates I_{KCa}, presumably by activating a membrane gating mechanism on a proximal K-channel.

Eckert and Tillotson (1978) were the first workers to directly correlate Ca^{2+} accumulation with I_{KCa} activation. Using the photoprotein aequorin, transient Ca^{2+} accumulation was measured in voltage-clamped giant neurones of the nudibranch *Anisodoris nobilis*. The activation kinetics and amplitude of I_{KCa} were related to the rate and extent of Ca^{2+} accumulation and the driving force on K^+.

Eckert *et al.* (1981) investigated the inactivation kinetics of I_{KCa} in molluscan neurones and in the protozoan *Paramecium*. Inactivation of the inward calcium conductance (g_{Ca}) in caesium-loaded cells (ensures blockade of potassium channels) depends upon the entry and accumulation of calcium during a depolarising pulse. During a prolonged pulse the inward calcium current declines, i.e. inactivates, to a new steady level. The time-course of removal of

inactivation in molluscan neurones, measured with arsenazo III, consists of two exponential components, a rapid component of 4.5ms and a slower component of 450ms (Thomas and Gorman, 1977). By removing the free intracellular Ca^{2+}, injection of EGTA slowed the rate of inactivation, increased the magnitude of the steady-state current response and increased the rate of removal of inactivation. Replacement of all or part of the external Ca^{2+} with barium ions (Ba^{2+}) also slowed inactivation because Ba^{2+} ions are not able to inactivate the Ca channel in the same way as Ca^{2+}. Using a double pulse technique, whereby pulse (I) amplitude and duration could be varied and immediately followed by a standard second pulse, showed that Ca^{2+} already present does not sum with the newly entering Ca^{2+} and facilitate I_{KCa}. Eckert *et al.* (1981) recognised that accumulation of Ca^{2+} on the inner surface of the cell membrane should reduce the driving force on the calcium influx and therefore reduce the magnitude of inward current. They concluded, however, that it is the absolute accumulation of Ca^{2+} at the inner membrane surface that is directly affecting the calcium channel gating. How does the I_{Ca} inactivation affect I_{KCa}? Using *Helix* bursting pacemaker neurones, known to have a strong I_{KCa} component, two identical voltage pulses were administered separated by a variable interval. As the interval was decreased the outward current response to the second pulse was reduced and recovered as the pulse interval was increased. Intracellular free Ca^{2+}, remaining from a prior Ca^{2+} entry, produces inactivation of I_{Ca} therefore the smaller the increase in internal Ca^{2+} concentration and hence a reduction in I_{KCa} during the second pulse (Eckert, Tillotson, and Brehm, 1981). This is schematically represented in Fig. 1.223 1 where an increase in intracellular Ca^{2+} concentration will initially increase g_{KCa} (step 1) and then

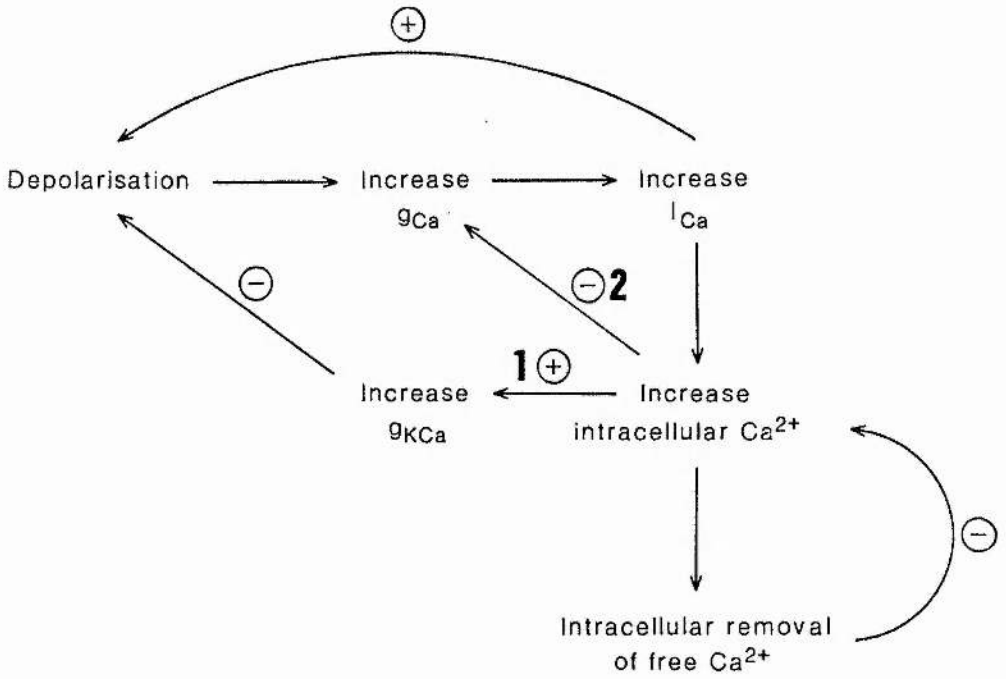


Fig. 1.223 1

The inter-relationship between intracellular calcium concentration and calcium-dependent conductances. An increase in intracellular Ca^{2+} has two opposing effects. Firstly an increase in g_{KCa} (step 1) and secondly a supression of any further increase in g_{Ca} (step 2) (adapted from Eckert, Tillotson and Brehm, 1981).

negatively feedback to suppress a further increase in gCa (step 2).

Once calcium traverses the membrane and enters the cytoplasm the complex buffering system regulates the magnitude and the time-course of its cytoplasmic accumulation (for review see McBurney and Neering, 1987). There are four major contributors to this homeostasis:

i) sequestration by mitochondria;

ii) sequestration by endoplasmic reticulum (ER);

iii) binding to cytoplasmic proteins e.g. calmodulin or paravalbumin and

iv) Ca^{2+} exchange across the cell membrane e.g. Na^{+}-dependent Ca^{2+} exchange or an ATP-dependent Ca^{2+} extrusion.

Although several of these mechanisms have been demonstrated in neurones their relative contributions to intracellular Ca^{2+} homeostasis is not clear (McBurney and Neering, 1987).

The advent of patch-clamping techniques has allowed further distinction between different types of I_{KCa}. There appears to be two main groups of K_{Ca}-channels:

i) Large unitary conductance (100-200pS), and

ii) Small unitary conductance (10-20pS)

(Latorre, Coronado and Vergara, 1984). The large conductance channel was originally reported in cultured bovine chromaffin cells (Marty, 1981) and later in a variety of preparations e.g. cultured rat myotubes (Pallota, Magleby and Barrett, 1981), clonal anterior pituitary cells (Wong, Lecar and Adler, 1982), bullfrog sympathetic neurones (Adams, Constanti, Brown and Clark, 1982), cultured mouse spinal neurones (Lambert *et al.*, 1984) and smooth muscle cells (Inoue, Kitamura and Kuriyama, 1985). Such a large conductance means that the openings and closings are quite obvious above the background noise level, hence these channels acquired the name "Big Brothers" or BK channels (Marty, 1983) and were also referred to as maxi channels

by Latorre and Miller (1983). These BK channels have been incorporated into phospholipid bilayers enabling the study of the channel molecules without other cellular components (Coronado and Labarca, 1984). There are at least three open states which may correspond to the binding of three Ca^{2+} near or in the channel (Barrett, Magleby and Pallotta, 1982).

The low conductance channel (K_s) was originally identified in *Helix* neurones by Lux, Neher and Marty, (1981) and later investigated by Hermann and Hartung (1982a; b). A similar low conductance channel has also been identified in smooth muscle cells (Inoue, Kitamura and Kuriyama, 1985) and *Aplysia* neurones (Hartung and Hermann, 1987). The K_s-channel can be activated by intracellular ionophoretic injection of Ca^{2+} (Herman and Hartung, 1982a). There is evidence that this channel is modulated by cAMP-dependent protein phosphorylation of a site closely associated with the channel (Ewald, Williams and Levitan, 1985). Protein phosphorylation caused a long-lasting increase (several minutes) in affinity of K_s-channels for Ca^{2+} and an increased probability of channel opening between -60mV and 100mV.

Although TEA^+ is classically considered to be an I_K channel blocker its action is not absolutely specific. Externally applied TEA^+ blocked both I_{KCa} and I_K channels in *Helix* neurones (Meech and Standen, 1975; Hermann and Gorman, 1981b). In addition, some workers have demonstrated that two coexisting components of I_{KCa} differ in their relative sensitivities to externally applied TEA^+ (Deitmer and Eckert, 1985; Inoue, Kitamura and Kuriyama, 1985). Tail current measurements from identified *Aplysia* neurones enabled the distinction between a large TEA^+-sensitive (1mM) component and a TEA^+-insensitive component in the same cell which may be affected by high

concentrations of TEA^+ (200mM). The relative contribution of these components varied between cell types. The reversal potential of the TEA^+-resistant tail current lay close to -60mV which is typical for I_{KCa} (Meech and Standen, 1975). A correlation has been demonstrated between the presence of a TEA^+-sensitive component and spontaneous bursting behaviour in *Aplysia* neurones (Gorman and Hermann, 1982). Those cells with only a TEA^+-resistant component are non-spontaneous.

Single channel recordings from smooth muscle membrane of rabbit portal vein show two different I_{KCa} conductances; 273pS (K_L-channel), and 92pS (K_S-channel) (Inoue, Kitamura, and Kuriyama, 1985). These channels are analogous to the aforementioned high and low conductance channels (Latorre, Coronado and Vergara, 1984). Externally applied TEA^+ (0.1mM) blocked activation of the K_L-channel but not that of the K_S-channel. Internal TEA^+ application was ineffective in blocking either channel. Studies with cultured rat muscle (Blatz and Magleby, 1984) showed that high concentrations of externally applied TEA^+ (10-100mM) reduced the macroscopic and unitary current (high conductance) amplitudes of I_{KCa}. In muscle the TEA receptor appears to be on the external surface of the membrane. Conversely Wong and Adler (1986) demonstrated that I_{KCa} from excised clonal anterior pituitary cells had TEA^+ receptors on the internal and external surface but I_{KCa} was more susceptible to internally applied TEA^+. In the light of these diverse findings it seems that there are at least two different types of I_{KCa} present in cell membranes which differ with respect to their unitary conductances and pharmacological sensitivities to applied TEA^+, the high conductance channel being more sensitive to externally applied TEA^+.

The fundamental requirement for I_{KCa} activation is Ca^{2+} availability therefore procedures or drugs which alter its availability will indirectly affect I_{KCa} (summarised in Table 1.223

1). Injection of EGTA (1-2mM) into the cytoplasm has been shown to block I_{KCa} activation via chelation of intracellular Ca^{2+} but does not prevent the Ca^{2+} influx (Connor, 1979; Pitman, 1979). Reduction of intracellular Ca^{2+} would increase the driving force on external Ca^{2+} ions thereby increasing the inward Ca^{2+} current. Under current-clamp conditions intracellular chelation with EGTA or citrate electrodes increases the Ca^{2+} entry and, combined with suppression of I_{KCa}, produces a calcium spike in a previously non-spiking identified insect motoneurone (Pitman, 1979).

A selection of externally applied metal cations compete with Ca^{2+} at binding sites and thereby prevent Ca^{2+} influx (Oyama, Akaike, Kuraoka, and Nishi, 1986). The polyvalent cations have a high binding affinity but a relatively low mobility and therefore block the Ca channel (Hagiwara and Byerly, 1981). Although there is some variation between preparations the rank potency of the polyvalent cations (in the mM range) is usually as follows (Hagiwara and Byerly, 1983):

$La^{3+} > Cd^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+} > Mg^{2+}$

At concentrations used experimentally, however, the cations may have additional effects upon the cell membrane such as modification of the cell surface charge and thereby interference with receptor binding (Oyama *et al.*, 1986).

A second class of blockers, the organic blockers, are more specific for certain I_{Ca} in the micromolar range (Hagiwara and Byerly, 1983). The phenylalkylamines, e.g. verapamil, and benzothiazepines, e.g. diltiazem, are somewhat less potent than the dihydropyridines, e.g. nifedipine and nitrendipine (Miller, 1985). Nitrendipine is nifedipine with a nitro group moved to the 3 position and one methyl ester converted to an ethyl ester (Hille, 1984).

Cinarizine and Fendiline are members of the diphenylalkylamine group (Gola and Ducreux, 1985). The phenylalkylamines and dihydropyridines are used clinically in the treatment of supraventricular cardiac arrhythmias and angina pectoris (Fleckenstein, 1977).

The phenylalkylamines such as verapamil (also known as D365, iproveratil and isoptin) and D600 (also known as methoxyverapamil) suppressed an inward Ca^{2+} current and increased the inactivation of a delayed outward current in *Helix* neurones (Kostyuk, Krishtal, and Doroshenko, 1975). They also suppress the outward current identified as I_{KCa} in the same preparation (Meech and Standen, 1974; 1975).

V cells are fast adapting *Helix* neurones with a large, overshooting, long-duration action potential. Gola and Ducreux (1985) have reported that at low concentrations (10-50μM) the phenylalkylamines and benzothiazepines prolong the V-cell action potential duration without blocking the Ca current underlying the upstroke of the spike and, under voltage-clamp, reduce the outward current without altering the inward Ca^{2+} current. In this system these blockers, at low concentrations, have a direct effect on the I_{KCa} channel which is not secondary to the suppression of I_{Ca}. At high concentrations (0.5-1.0μM) the spike was reversibly abolished indicating eventual blockade of I_{Ca}. D600 was the most effective channel blocker in this system while nifedipine and cinarizine (10-100μM) had no detectable blocking effect.

Studies using [^{3}H] nitrendipine and [^{3}H] desmethoxyverapamil have shown that the dihydropyridine binding site on the calcium channel is separate from the phenylalkylamine and benzothiazapine binding site (Miller, 1985).

Anticonvulsant concentrations (mM range) of barbiturates caused broadening of the action potential in leech Retzius neurones (Kleinhaus and Pritchard, 1977a) and leech sensory neurones

(Kleinhaus and Pritchard, 1977b). The authors suggest that the drugs interfere with the repolarisation process by blocking an inward Ca^{2+} current which would activate a K^+ conductance. Pentobarbital and methohexital (0.1-1.0mM) also caused broadening of the action potential. High external Ca^{2+} reversed this effect whereas low concentrations favoured the blockade. The drug effect was greatest in cells which had a large Ca^{2+} conductance (Kleinhaus and Pritchard, 1977b). The results were consistent with a barbiturate block of I_{KCa}. More recent evidence confirms that barbiturates block divalent cation entry into leech nociceptive neurones (Johansen and Kleinhaus, 1986). It seems that blockade of I_{KCa} by barbiturates is a secondary effect due to suppression of I_{Ca}.

The suppression of I_{KCa} by the drugs so far reviewed has been an indirect response to the direct blockade of I_{Ca} therefore many compounds which specifically block I_{Ca} will in turn block I_{KCa}. More recent interest has focussed on particular toxins extracted from animal venoms which specifically block I_{KCa}, namely apamin and charybdotoxin.

The best characterised type of I_{KCa} is the slow conductance one responsible for the after hyperpolarisation (AHP) that follows action potentials in rat skeletal muscle cells (Hugues, Schmid, Romey, Duval, Frelin, and Lazdunski, 1982). This channel is specifically blocked with a high affinity by a toxin called apamin (Lazdunski, 1983; Romey, Hugues, Schmid-Antomarchi and Lazdunski, 1984). In addition apamin-sensitive channels have also been reported in hepatocytes (Burgess, Claret and Jenkinson, 1981), neuroblastoma cells (Hugues, Romey, Duval, Vincent, Lazdunski, 1982), smooth muscle (Banks, Brown, Burgess, Burnstock, Claret, Cocks and Jenkinson, 1979) and rat pheochromocytoma cells (Schmid-Antomarchi, Hugues and

Lazdunski, 1986).

Apamin was first extracted from honeybee venom by Habermann (for a review see Habermann, 1972). It is a polypeptide chain of 18 amino acids with two disulphide bridges (see Appendix IIIB for the structure). Structure-activity relationship studies indicate that the two adjacent argenine residues at positions 13 and 14 are an essential part of the binding site and therefore of toxin action (Lazdunski, 1983). Romey and Lazdunski (1984) established that the previously reported BK channels in rat myotubules (Pallotta, Magleby and Barrett, 1981) which are TEA^+-sensitive are insensitive to apamin. The apamin-sensitive macroscopic I_{KCa} currents were shown to be TEA^+-insensitive. There were, however, no single channel recordings of apamin-sensitive/TEA^+-insensitive channels in this study. There appears to co-exist two distinct classes of I_{KCa} in the rat muscle preparation which exhibit different sensitivities to internal calcium between membrane potentials of -80 and -50mV. High internal calcium activates the TEA^+-sensitive I_{KCa} and inhibits the apamin-sensitive I_{KCa}. Furthermore, the apamin-sensitive channel is more sensitive to low concentrations of intracellular Ca^{2+} ions. Romey and Lazdunski (1984) did not offer any explanation of why internal Ca^{2+} should block I_{KCa}. The inhibition of I_{KCa} by internal Ca^{2+}, however, may reflect the accumulation of Ca^{2+} at the inner membrane surface preventing further increase in the inward I_{Ca} (Eckert, Tillotson and Brehm, 1981). It is possible the K channel may be directly blocked by a "threshold" level of internal Ca_{2+}, though there is no evidence for this.

These findings were confirmed by more recent studies on cultured skeletal muscle where single channel recordings have shown a class of small conductance (10-14pS) I_{KCa} channels (K_S channels) which are apamin-sensitive and TEA^+-insensitive (Blatz and Magleby, 1986). In

addition, at more negative membrane potentials (-60 to -20mV), the K_S channels are more sensitive to internal Ca^{2+} and are therefore activated before BK channels. Apamin appears to be highly specific for a particular subclass of I_{KCa}. Romey and Lazdunski (1984) suggest that the physiological role of the low conductance apamin-sensitive channel is to participate in AHP, whereas the high conductance TEA^+-sensitive channel may serve to prevent prolonged depolarisations that would elevate the internal Ca^{2+} concentration.

Autoradiographs of ^{125}I-apamin binding sites on rat brain slices show specific labelling of discrete areas in the cortex (Mourre, Schmid-Antomarchi, Hugues, and Lazdunski, 1984; Mourre, Hugues and Lazdunski, 1986). In addition, there exists an endogenous apamin-like factor which has been isolated from pig brain (Fosset, Schmid-Antomarchi, Hugues, Romey, and Lazdunski, 1984). This, so far uncharacterised, peptide mimics the action of apamin, is sufficiently similar in structure to apamin to be recognised by anti-apamin antibodies and , like apamin, is destroyed by trypsin and not chymotrypsin. The physiological role of this apamin-like factor is speculative. Nevertheless, blocking a component of repolarisation may alter the action potential shape or increase the spike frequency. Perhaps the factor is released from nerve terminals as a neuromodulator. Whatever its role, the factor is likely to be present in other species including man.

Myotonic muscular dystrophy is a condition characterised by muscle stiffness and hampered relaxation after voluntary contractions. This is an inherited disease which results in alterations in the muscle membrane characteristics. Renaud *et al.* (1986) reported that muscle membranes from afflicted patients possess apamin-sensitive receptors whereas healthy subjects do not have the

receptor. The presence of an apamin-sensitive I_{KCa} channel and a less negative resting potential result in repetitive burstings of activity which give the condition its name (Renaud, Desnuelle, Schmid-Antomarchi, Hugues, Serratrice and Lazdunski, 1986)

Another toxin has been discovered which reversibly blocks the large BK I_{KCa} conductances from rat muscle tubules inserted into planar phospholipid bilayers (Miller, Moczydlowski, Latorre and Phillips, 1985; Anderson, MacKinnon, Smith and Miller, 1988; MacKinnon and Miller, 1988). This toxin, charybdotoxin (CTX), has been extracted from the venom of the Israeli scorpion, *Leiurus quinquestiatus*. Application of apamin had no effect upon these BK channels confirming that CTX operates on a different class of I_{KCa}. Anderson *et al.* (1988) demonstrated that CTX binds to the open and closed channel in a simple bimolecular reaction and that dissociation of CTX from the channel is enhanced by high internal K^+ concentrations depolarisation (MacKinnon and Miller, 1988). The results indicated that CTX physically plugs the K channel from the external surface of the membrane (MacKinnon and Miller, 1988). CTX has also been reported to block I_{KCa} in bullfrog ganglion neurones (Pennefather and Goh, 1987).

The fruit fly *Drosophila melanogaster* has a genetic defect called a "slow poke" (slo) mutant. The fast calcium-dependent outward current (previously called I_{Acd} by Salkoff, 1983a) is completely abolished by these mutation. The slo mutant flight muscles have prolonged Ca^{2+} spikes suggesting a major role for I_{Acd} in muscle action potential repolarisation (Ganetzky and Wu, 1985).

A group of molluscan neurones called "burster" neurones are characterised by their pattern of firing. Such neurones generate endogenous bursts of action potentials separated by periods of quiescence in which the membrane becomes hyperpolarised (Meech,

1979). The interburst hyperpolarisation gives way to a slow depolarisation and another train of action potentials. The mechanism underlying these bursts involves cyclical variations in intracellular ionised calcium which activate an outward current carried by K^+ (Meech and Standen, 1975; Meech, 1979). The mechanism is that during rapid action potentials, Ca^{2+} ions enter the cell and accumulate. This accumulation has been measured with arsenazo III (Thomas and Gorman, 1977; Gorman and Thomas, 1980a). After a sufficient rise in intracellular Ca^{2+}, I_{KCa} is activated which causes the slow hyperpolarisation which in turn prevents further Ca^{2+} entry. Intracellular Ca^{2+} falls, so reducing I_{KCa} and allowing depolarisation leading to the next burst of action potentials. The dependence of I_{KCa} upon intracellular Ca^{2+} renders the channel susceptible to other factors such as hormones or synaptic modulators which modify cellular calcium metabolism (Hille, 1984).

In summary, I_{KCa} has a similar time course of activation as I_K but requires calcium for its activation. At least two types of I_{KCa} have been distinguished. The first is activated by intracellular Ca^{2+}, and shows a relatively slow time course and is thought to underly post-activity AHP in molluscan and vertebrate neurones. The second is activated by Ca^{2+} crossing the membrane, shows a faster time course of activation and is depressed by accumulation of intracellular Ca^{2+}. Procedures or drugs which block Ca^{2+} availability will indirectly affect I_{KCa}, whereas the animal toxins specifically block two subclasses of I_{KCa} (see Table 1.223 1). Single channel recordings also show two channel sub-types with different kinetics and pharmacological profiles (see Table 1.223 2). The relationship between the classes of macroscopic currents and unitary channels is, however, unclear at present.

TABLE 1.223 1

Pharmacological agents which block I_{KCa}.

INORGANIC BLOCKERS

Metal Cations	$La^{3+} > Cd^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+} > Mg^{2+}$

References: 1, 2 & 3.

ORGANIC BLOCKERS

Benzothiazepines	Diltiazem
Dihydropyridine	Nifedipine
	Nitrendipine
Phenylalkylamines	Verapamil
	D600
Diphenylalkylamines	Cinarizine
	Fendiline

References: 1, 2, 4 & 5.

TOXIN BLOCKERS

Apamin

References: 6, 7, 2 & 8.

Charybdotoxin

References: 9, 10 & 11.

References:

1. Oyama, Akaike, Kuraoka and Nishi, 1986.
2. Oyama and Kuraoka, 1986.
3. Hagiwara and Byerly, 1983.
4. Miller, 1985.
5. Gola and Ducreux, 1985.
6. Schmid-Antomarchi, Renaud, Romey, Hugues, Schmid and Lazdunski, 1985.
7. Traore, Cognard, Potreau and Raymond, 1986.
8. Blatz and Magleby, 1986a.
9. Miller, Moczydlowski, Latorre and Phillips, 1985.
10. Pennefather and Goh, 1987.
11. Anderson, MacKinnon, Smith and Miller, 1988.

TABLE 1.223 2

Pharmacological and electrophysiological characteristics of the K_{Ca}-channel.

SMALL CONDUCTANCE	References:
(10-20pS)	1,2,3 & 4.
K_S-channel	11.
Apamin-sensitive	12 & 13.
TEA^+-insensitive	11,12,13 & 15.
no data for CTX-sensitivity	

LARGE CONDUCTANCE	References:
(100-200pS)	5,6,7,8 & 9.
KB-channel	10.
K_L-channel	11.
Apamin-insensitive	14.
TEA^+-sensitive	11 & 15.
CTX-sensitive	14,16 & 17

References:

1. Lux, Neher and Marty, 1981.
2. Hermann and Hartung, 1982a.
3. Hermann and Hartung, 1982b.
4. Hartung and Hermann, 1987.
5. Marty, 1981.
6. Pallota, Magleby and Barret, 1981.
7. Wong, Lecar and Adler, 1982.
8. Adams, Constanti and Brown, 1982.
9. Lambert, Cottrell, Peters, Green and Newton, 1984.
10. Marty, 1983.
11. Inoue, Kitamura and Kuriyama, 1985.
12. Romey and Lazdunski, 1984.
13. Blatz and Magleby, 1986a.
14. Miller, Moczydlowski, Latorre and Phillips, 1985.
15. Deitmer and Eckert, 1985.
16. Pennefather and Goh, 1987.
17. Anderson, MacKinnon, Smith and Miller, 1988.

1.224 Slow-outward (I_{Kslow}).

A slow outward current (SOC) has been described in *Aplysia* neurones which is thought to regulate firing frequency in response to prolonged stimulation (Cote, Zbicz and Wilson, 1978). Barbiturates at low concentrations (0.05-0.5mM) suppressed the ability of these *Aplysia* neurones to fire in response to prolonged depolarisation (Cote, Zbicz and Wilson, 1978). Once the cell had undergone rapid adaptation it could be made to spike by increasing the depolarising current, indicating that the spike mechanisms had not inactivated. Voltage-clamp studies showed that pentobarbital and phenobarbital induced a slow outward current which is activated at potentials more negative than -40mV and increased with depolarisation. Low potassium saline enhanced the effect of the barbiturates. Altering the external K^+ concentration in the presence of the barbiturates gave a +38mV shift in SOC reversal potential for a 10-fold change. Barbiturates seem to enhance a slow outward current (SOC) which is at least partly responsible for spike frequency adaptation. This effect of barbiturates on the SOC contrasts with their blockade of I_{KCa} in leech neurones (Kleinhaus and Pritchard, 1977a; 1977b).

Pharmacologically the slow outward current is unlike those already reported in molluscan neurones (Thompson, 1977). The net outward and tail currents were only slightly reduced with externally applied 4-AP while TEA^+ at high concentrations (100μM) almost halved the response (Huguenard, Zbicz, Lewis, Evans and Wilson, 1985). Barium (10μM) or muscarine (35μM) had no measureable effect indicating that this current is unlike that described by Brown and Adams (1980). Where muscarine agonists turn off an outward current called an M-current. Oubain had no effect on the SOC suggesting that the electrogenic pump is not involved in this current. External Na^+ and Cl^- manipulations had no significant effect. Reduction of

external Ca^{2+} or Ca-channel blockers did, however, reduce the SOC during prolonged exposure though it is unclear whether these effects are secondary to non-specific membrane destabilisation or an actual Ca^{2+} dependency (Huguenard *et al.*, 1985). The mitochondrial uncoupling agent DNP and an inhibitor of intracellular Ca^{2+} sequestration, mersalyl acid, both elevated intracellular Ca^{2+} and enhanced SOC. Surprisingly, intracellular EGTA injection only slightly reduced the SOC. An influx of Ca^{2+} is not necessary for SOC activation, but intracellular Ca^{2+} appears to exert some modulatory role in SOC activation. The SOC, however, is not identical to the classical I_{KCa} observed after brief bursts of action potentials in molluscan neurones (Meech and Standen, 1974; 1975).

In summary, the SOC is a novel potassium current which is enhanced by low concentrations of barbiturates and blocked by externally applied TEA^+. Calcium channel blockers also reduce the SOC and, though this current is not dependent on Ca^{2+} entry into the cell, intracellular Ca^{2+} may have a modulatory effect.

1.225 Sodium-Activated (I_{KNa}).

In view of previous work demonstrating the influx of ions regulating channel conductances, recent authors have reported the presence of a late sodium-activated potassium channel in mammalian cardiac cells (Kameyama, Kakei, Sato, Shibasaki, Matsuda and Irisawa, 1984), cultured avian neurones (Bader, Bernheim and Bertrand, 1985), and crayfish neurones (Hartung, 1985). Single channel recordings show that this channel has a large conductance of 220pS and channel opening probability is dependent upon the internal Na^+ concentration and is unaffected by internal Ca^{2+} (Kameyama *et al.*, 1984). These observations were supported by voltage-clamp studies in the crayfish (Hartung, 1985). The outward current was reduced if tetrodotoxin (TTX) was present externally or in Na^+-free saline, but not when Li^+ was substituted for Na^+. The potassium channel blockers TEA^+ and 4-AP also depressed the outward current (Bader *et al.*, 1985; Hartung, 1985).

Bader *et al.* (1985) suggested that this channel can probably be triggered by sodium entering the neurone during a single action potential i.e. only a small elevation in Na^+ is necessary. The activation of this conductance will therefore hasten the decay of the inward current. Its fast activation kinetics mean that it can participate in rapid repolarisation which may affect the duration of the action potential. Elevation of internal sodium by any other means e.g. sodium-mediated, excitatory synaptic events, will also affect the excitability of the neurone. Although the characteristics of I_{KNa} theoretically enable it to play a physiological role, its role in neuronal function has not been elucidated (Hartung, 1985).

1.226 ATP-Regulaton.

Channels controlled by nucleotides are a relatively recent discovery. The best characterised channel whose activity is controlled by ATP is a class of potassium channel. The binding of ATP to these channels induces channel closure (Stanfield, 1987). Norma (1983) described for the first time the presence of ATP-dependent K channels in cardiac muscle. ATP-dependent channels were subsequently found in skeletal muscle (Spruce, Standen and Stanfield, 1985; 1987) and in ß-cells of pancreatic islets (Ashcroft, Harrison and Ashcroft, 1984).

1.2261 Cardiac cells.

The ATP-dependent K channels in cardiac muscle were closed by physiologically high concentrations (3-4mM) of the nucleotide present during the resting state (Norma, 1983). AMP had no effect on channel activity. Hypoxia, low intracellular ATP and cyanide treatment caused these K channels to open. Insufficient blood flow to the heart, otherwise known as cardiac ischaemia, results in the lowering of muscle metabolism and, by inference, a reduction in ATP levels. Under these conditions ATP-regulated channels contribute to the increase in outward current and shortening of the cardiac action potential (Norma and Shibasaki, 1985).

1.2262 Skeletal muscle.

The skeletal muscle ATP-dependent K channels were also found to be voltage-dependent, the open state probability increasing with depolarisation. The single-channel conductance was 15pS in physiological K^+ concentrations (Standen, Stanfield and Ward, 1985). The binding of ATP to these channels and subsequent closure did not require hydrolysis of ATP to ADP. Furthermore, the binding of ATP is independent of Mg^{2+}. Non-hydrolysable ATP analogues such as adenylylimidodiphosphate (AMP-PNP), were as effective as ATP in closing channels. In addition, ADP and AMP are able to close the

channel but are much less effective than ATP. Changing the adenosine base to guanosine (GTP), inosine (ITP) or xanthosine (XTP) also resulted in a much reduced efficacy. Interestingly, no structural alterations to the nucleotide base completely abolished its ability to modulate channel activity. The channel was blocked by internal and external application of TEA^+ (2mM) (Standen, Stanfield and Ward, 1985; Spruce, Standen and Stanfield, 1987).

The ATP-dependent channels are present at high density in frog skeletal muscle, a density similar to, or higher than, that found for I_K channels (Spruce, Standen and Stanfield, 1985; Standen, Stanfield and Ward, 1985). It is highly unlikely that this prevalence is merely biological redundancy and instead suggests an important physiological role for this channel. Under normal conditions the intracellular ATP concentration (5mM) is sufficiently high to close all the ATP-dependent channels. Spruce *et al.* (1987) suggested that these channels open during prolonged muscle contractions to allow K^+ efflux in a manner that is related to energy usage. However, K^+ is known to be a powerful vasodilator, which would link increased blood flow in muscle arterioles with increased energy usage accompanying muscular activity (Spruce, Standen and Stanfield, 1987).

1.2263 Pancreatic ß-cells.

In the endocrine pancreatic ß-cells, stimulation with glucose or glyceraldehyde evokes membrane depolarisation due to closure of ATP-dependent K^+ channels which results in the release of insulin (Ashcroft, Harrison and Ashcroft, 1984; Cook and Hales, 1984; Rorsman and Trube, 1985; Misler, Falke, Gillis and McDaniel, 1986; Petersen, Findlay, Suzuki and Dunne, 1986). Ashcroft *et al.* (1984) called these channels G channels because of their responsiveness to glucose. In contrast to the ATP-sensitive K channels of skeletal

muscle (Spruce, Standen and Stanfield, 1985), the ß-cell K channels are relatively insensitive to changes in membrane potential (Petersen, Findlay, Suzuki and Dunne, 1986) and have a resting conductance of 60pS (Trube, Rorsman and Ohno-Shosaku, 1986). The drug tolbutamine (0.1mM) induces hypoglyceamia and has been shown to achieve this channel closure and presumably release of insulin. The drug diazoxide (0.4mM) induces hyperglyceamia and was able to counteract the effect of glucose or tolbutamine by opening channels and thereby preventing insulin release (Trube, Rorsman and Ohno-Shosaku, 1986). One possible functional significance is that the ATP generated during glycolysis is responsible for channel closure. In the glycolytic pathway the transfer of a phosphate group from 3-phosphoglyceroyl phosphate to ADP to produce ATP is catalysed by the membrane-bound enzyme phosphoglycerate kinase (PGK). ATP generated from this reaction could act locally at the inner membrane surface to close the resting K^+ channel (Petersen, Findlay, Suzuki and Dunne, 1986).

The general concept is that an ATP-dependent K channel would form a link between the metabolic status of the cell and its permeability. This biochemical link does not amplify the effect of ligand binding. So far there have been no reports in the literature of ATP-dependent K channels occurring in neuronal membranes.

1.227 Voltage-Dependent Transmitter-Mediated Potassium Currents.

Conventionally the opening and closure of receptor-linked ion channels has been considered independent of membrane voltage. Thus, from Ohm's law, the current flowing through such a channel will be directly proportional to the driving force on the permeant ions for a range of membrane potentials (see Eq. 1.125). In some receptor-linked channels the current flowing through the membrane will now be influenced by the membrane potential. These voltage-sensitive receptor-linked ion channels have been found in a number of vertebrate and invertebrate preparations. The following section will focus on voltage-dependent transmitter mediated potassium currents (Table 1.227 1).

1.2271 Gill withdrawal reflex of *Aplysia*.

The gill-withdrawal reflex of the marine mollusc *Aplysia* has been used to study the cellular basis of behavioural sensitisation. The siphon is a tubular extension of the outer mantle situated on the dorsal surface of the animal and this is responsible for the expulsion of seawater from the mantle cavity. The gill is located near the dorsal surface underneath the mantle shelf, is highly folded with a rich blood supply and is responsible for the gaseous exchange between the blood and fresh seawater (Kandel, 1979).

Sensitisation is defined as the prolonged enhancement of a behavioural response to one stimulus as a result of the presentation of another stimulus, typically a noxious or novel one. In this case tactile stimulation of the siphon and consequent withdrawal of the gill undergoes prolonged sensitisation following a strong stimulus to the head (Klein and Kandel, 1978; 1980). Sensitisation could be achieved by stimulating the appropriate nerve pathway from the head. Nerves from the sensitisation pathway then synapse onto the presynaptic terminals of the siphon sensory neurones.

The cellular mechanism is a prolonged increase in transmitter release from the presynaptic terminals of the sensory neurones innervating the siphon i.e. presynaptic facilitation. Applied 5-hydroxytryptamine (5-HT or serotonin) mimicked stimulation of this pathway and prolonged the duration of the action potential recorded in the cell body. It was initially thought that Ca^{2+} was the current carrier in this system (Klein and Kandel, 1978) but voltage-clamp studies have demonstrated that a conductance decrease in potassium occurs (Klein and Kandel, 1980). This effectively delays repolarisation and so prolongs the inward calcium current thereby enhancing transmitter release. The serotonin-sensitive current was termed I_S or S-current.

Further single channel studies on these 5-HT-sensitive K channels have identified a novel K channel which shares many of the features of the macroscopic I_S (Siegelbaum, Camardo and Kandel, 1982 ; Siegelbaum, Belardetti, Camardo and Shuster, 1986). This channel is activated at the resting potential of *Aplysia* sensory neurones, its activity is only moderately voltage-dependent and it is insensitive to changes in intracellular Ca^{2+}. Application of 5-HT caused a dose-dependent increase in the cell input resistance and a reduction in the number of active channels. The transmitter appears to cause the individual channels to close for prolonged times in an all-or-nothing manner. In addition 5-HT decreased the channel activity when it was applied outside the *in situ* patch. Considering the high resistance pipette-membrane seal this suggests the involvement of a second messenger. This was confirmed by intracellular injection of cAMP which decreased channel activity in a similar way to 5-HT (Shuster, Camardo, Siegelbaum and Kandel, 1985).

Recent investigations have shown that the peptide Phe-Met-Arg-

Pheamide (FMRFamide) controls the gating of the S-channel by causing an increase in the open channel probability (Belardetti, Kandel and Sieglebaum, 1987). This is achieved via a second messenger which is distict from cAMP (Belardetti *et al.*, 1987). The hyperpolarising action of FMRFamide opposes that of 5-HT and offers a further modulatory pathway for this system.

The pharmacological profile of the S channel is similar to that of I_S. External application of TEA^+ (10mM) produced a small but consistent decrease in the single channel current amplitudes (Shuster and Sieglebaum, 1987) and macroscopic currents (Klein, Camardo and Kandel, 1982). External TEA^+ blockade is, however, moderately voltage-dependent (Sieglebaum, Camardo and Kandel, 1982). External application of 4-AP, apamin or Co^{2+} had no effect upon S channels in this preparation (Sieglebaum, Camardo and Kandel, 1982; Shuster and Sieglebaum, 1987). Though external Co^{2+} did reduce the magnitude of the macroscopic S current, this action was thought to be due to Co^{2+} interefering with the binding of 5-HT to the receptor (Klein *et al.*, 1982).

In summary, presynaptic facilitation is achieved by 5-HT closing a novel K channel on the pre-synaptic terminal, thereby prolonging the inward Ca current which in turn would lead to increased transmitter release. In the intact animal this mechanism results in facilitation of the sensitisation reflex and is thought to provide the cellular basis for a simple form of learning.

1.2272 **Frog sympathetic ganglion cells.**

Bullfrog sympathetic ganglia are comprised of two different classes of neurone. These cells, termed B and C, receive different inputs and respond with different slow synaptic potentials. Orthodromic stimulation of preganglionic fibres evokes three separate post synaptic responses in B cells; fast, slow and late-slow excitatory post synaptic potentials (epsps) (Adams, Jones, Pennefather, Brown, Koch and Lancaster, 1986). In contrast to other epsps, the input resistance was increased during the slow epsp indicative of a conductance decrease. Hyperpolarising current reversed the event to a hyperpolarising potential (Weight and Votava, 1970). Furthermore, application of muscarinic agonists produced the slow epsp and increased the tendency toward repetitive spike discharges (Kuba and Koketsu; 1976; Weight and Votava, 1970).

This muscarine-sensitive current, or M-current (I_M), was further investigated under voltage-clamp (Brown and Adams, 1980; Brown, Constanti and Adams, 1981; Adams, Brown, Constanti, 1982a; Akasu, Gallagher, Kokestu and Shinnick-Gallagher, 1984). M channels are closed at membrane potentials more negative than -60mV. Between potentials -60 and -10mV, I_M was activated and produced a steady outward current and, since it did not show any time-dependent inactivation, it contributed to the resting potential within this range (Adams, Brown and Constanti, 1982a). The reversal potential of the current lay between -70 and -100mV and gave a predicted Nernstian shift for a ten-fold change in external K^+, indicating that potassium is the major charge carrier (Brown, Constanti and Adams, 1981).

Muscarinic agonists or synaptically released ACh turn off the M-current to produce the characteristic slow depolarisation (Adams and Brown, 1982). The action of muscarine was not potentiated by TEA^+ (10mM) or 4-AP (1-5mM) (Brown and Adams, 1980) which are known to

block some K channels in other preparations. Divalent cations known to block Ca^{2+} currents had no blocking effect on I_M (Adams, Brown and Constanti, 1982b). The muscarinic response can be completely blocked by 10-100nM atropine (Adams, Jones, Pennefather, Brown, Koch and Lancaster, 1986). Thus the M-current is an outward K^+ current activated as the membrane potential becomes depolarised. This would effectively oppose depolarisations and act as a "gain control" for other synaptic inputs. That is, during depolarisation an opposing outward current would reduce neuronal excitability and limit the firing frequency of the neurone.

Orthodromic stimulation of preganglionic fibres elicits three separate post-synaptic responses in C cells : fast epsp, fast ipsp and a late slow epsp (Adams *et al.*, 1986). An LHRH-like peptide has been reported to be released from preganglionic C fibres in the frog (Jan, Jan and Kuffler, 1979). LHRH-like immunoreactivity is confined to the spinal pathways which, when stimulated, produce late slow epsps. More recent investigations have confirmed that the preganglionic fibres synapsing onto the C cell co-release LHRH-like peptide with ACh. In this system LHRH-like peptide has two separate effects. Repetitive stimulation of the C axons caused a late slow epsp in B cells, although it is somewhat slower in B cells than C cells (Jan and Jan, 1982). The B cell response is mediated by the LHRH-like peptide which diffuses in the extracellular space from C axons to proximal B cells, whereupon the LHRH-like peptide selectively depresses I_M and the I_{AHP} (Jan and Jan, 1982). In the B cells there are two distinct types of calcium-activated potassium current (Pennefather, Lancaster, Adams and Nicoll, 1985). The first is a rapid voltage-dependent I_{KCa} which can be blocked by CTX (Pennefather and Goh, 1987). The second is termed I_{AHP}, and

underlies the late slow epsp (Brown, Constanti and Adams, 1981) and can be partially blocked by apamin (Adams *et al.*, 1986).

The muscarinic and peptidergic responses recorded from the same B cell have a similar voltage-dependency and conductance decrease. Activation of these two different receptors are thought to control shared ionic mechanisms in sympathetic ganglion cells (Kuffler and Sejnowski, 1983). Under physiological conditions the I_M and I_{AHP} act synergistically to prevent repetitive firing during maintained stimuli (Adams *et al.*, 1986).

In summary, the M-current is an outward K^+ current activated as the membrane potential becomes more depolarised. Muscarinic agonists or synaptically released ACh turn off the currrent resulting in a slow depolarisation.

1.2273 Molluscan Peptide FMRFamide.

Cottrell (1982) reported that the molluscan peptide FMRFamide (Phe-Met-Arg-Phe-NH_2) has opposing dual effects on *Helix* neurones which are dependent upon membrane voltage. At the resting potential application of the peptide hyperpolarised the cell. The magnitude of hyperpolarisation increased when external K^+ was lowered but remained unaffected by alterations in Cl^- concentration. This outward current was reduced, but not completely abolished, by external TEA^+ (100mM). The results indicated that the hyperpolarisation was due to an increase in K conductance which had the properties of I_K.

At potentials more positive than the resting potential, the peptide elicited an apparent inward current which increased in magnitude as the cell was depolarised. Substitution of Ca^{2+} with Co^{2+} or Ba^{2+} reversibly abolished the response, while intracellular ionophoresis of Ca^{2+} augmented the response. The results implied an indirect role of Ca^{2+}. The apparent inward current at relatively depolarised potentials could result from the suppression of a calcium-activated potassium current (Cottrell, 1982; Cottrell, Davis and Green, 1984). Subsequent investigations have shown single channel currents with the characteristics of K channels which appeared less frequently on FMRFamide application (Cottrell and Green, 1984).

The physiological significance of this dual action by one transmitter is not yet known. It has, however, been suggested by Cottrell (1982) that the two effects occur on different axonal processes and may result in suppression and potentiation of transmitter release.

TABLE 1.227 1.

Voltage-Dependent Transmitter Mediated g_K.

PREPARATION	TRANSMITTER	EFFECTS	MECHANISM
Mammalian cortical neurones.	ACh (M)	Excitation.	Reduced g_K
References: 1			
Frog sympathetic ganglia cells.	ACh (M)	Repetitive firing.	Reduced g_K
	LHRH-like peptide.	Long-lasting epsp.	
References: 2, 3, & 4.			
Gill-withdrawal reflex.	5-HT cAMP	Sensitization. Pre-synaptic facilitation.	Reduced g_K
	FMRFamide		Increased g_K
References: 5, 6, 7 & 8.			
Snail neurones.	FMRFamide	Hyperpol. Inward current.	Increased I_K Decreased I_{KCa}
References: 9 & 10.			

References:

1. Krnjevic, Pumain and Renaud, 1971.
2. Kuba and Koketsu, 1976.
3. Brown and Adams, 1980.
4. Brown, Constanti and Adams, 1981.
5. Klein and Kandel, 1978.
6. Klein and Kandel, 1980.
7. Siegelbaum, Camardo and Kandel, 1982.
8. Belardetti, Kandel and Siegelbaum, 1987
9. Cottrell, 1982.
10. Cottrell, Davis and Green, 1984.

1.228 Anaesthetic-activated potassium current ($I_{K(An)}$).

A recent report by Franks and Lieb (1988) has identified a novel neuronal K^+ current which was activated by volatile anaesthetics employed at surgical partial pressures. One neurone in the parietal ganglion of the pond snail *Lymnaea stagnalis* was particularly sensitive to anaesthetics and underwent large hyperpolarisations which suppressed its normal spontaneous activity in the presence of the anaesthetic. The effect was reversed when the anaesthetic was removed. Despite identifying and blocking I_A, I_K and I_{KCa}, anaesthetics still evoked a current which reversed around command potentials of -80mV and was insensitive to external chloride removal. A four-fold increase in external K^+ gave a shift in reversal potential close to that predicted by the Nernst equation and so the current was termed $I_{K(An)}$. Furthermore, there was a direct correlation between the presence of $I_{K(An)}$ in a neurone and the ability of anaesthetics to inhibit its spontaneous activity, in that not all of the spontaneously active neurones were sensitive to anaesthetics.

1.23 PROTON CURRENT (I_H).

Protons are not passively distributed across the cell membrane, the cytoplasm is maintained more alkaline than one would predict according to the membrane potential and equilibrium potential for H^+ (Thomas, 1977). Thomas and Meech (1982) indicated that H^+ might be a charge carrier in its own right. Using *Helix* neurones under voltage-clamp, a voltage-dependent outward H^+ current was observed at depolarised potentials. The H^+ currents were originally considered "non-specific" in that they flowed through several different ion channels. Further investigations by Meech and Thomas (1987) confirmed the presence of a voltage-activated H^+ current. Byerly and Hagiwara (1982) also described a non-specific outward current in perfused *Lymnaea* neurones which has similar characteristics to the current described by Thomas and Meech (1982). A more recent study by Byerly, Meech and Moody (1984) demonstrated a unique H^+ channel which is highly selective for protons in *Lymnaea* neurones. The outward H^+ currents were reduced by high intracellular pH, low extracellular pH, external Cd^{2+} and 4-AP. Byerly *et al.* (1984), however, did not consider an OH^- component. The product of pH and pOH must equal the negative log of the dissociation constant of water i.e. 14, therefore hydroxyl ions must be present and should not be ignored.

A H^+ current has also been studied in the immature oocyte of the urodele amphibian *Ambystoma* (Barish and Baud, 1984). At depolarised potentials the H^+ current constituted the major portion of the outward current. The current reversed around +10mV and an external change in pH between pH 6.9 and 8.4 units produced a shift in the reversal potential predicted by the Nernst equation. TEA^+ had no effect upon this current. The activation and inactivation kinetics of the current each follow a single exponential time-course.

The physiological significance of this channel in molluscan

neurones is that it compensates for the rapid intracellular acidification generated by trains of action potentials. Influx of Ca^{2+} during action potentials produces a local increase in free intracellular Ca^{2+} at the cell membrane and is restored to resting level by Ca^{2+}/H^{+} exchange at intracellular sites (Ahmed and Connor, 1980). The similarities between the molluscan H^{+} current and the more slowly developing H^{+} current of vertebrate oocytes suggests that this may be a widely distributed mechanism for internal pH regulation (Meech and Thomas, 1987).

1.24 CHLORIDE CURRENTS.

In many animal cells chloride is distributed so that the chloride equilibrium potential (E_{Cl}) is near the resting potential. Thus, in such cells the Cl channels will, like K channels, oppose membrane depolarisations by allowing a Cl^- influx into the cell.

Chloride channels can be divided into three main categories: transmitter-activated synaptic Cl channels, voltage-dependent chloride channels and ion-dependent channels. Transmitter-activated channels (inhibitory synapses mediated by gamma-aminobutyric acid (GABA) and glycine) will not be reviewed. Instead emphasis will be placed upon the other two categories.

1.241 Voltage-dependent chloride current(I_{ClV}).

Voltage-dependent chloride channels have been reported in a variety of cells e.g. hippocampal pyramidal cells (Madison, Malenka and Nicoll, 1986), luminal membrane of cultured pulmonary alvelar cells (Schneider, Cook, Gage and Young, 1985), *Aplysia* neurones (Chesnoy-Marchais, 1983; Chesnoy-Marchais and Evans, 1986), rat superior cervical ganglion cells (Selyanko, 1984) and *Torpedo* electroplaque membranes (White and Miller, 1979; 1981; Hanke and Miller, 1983). To study these currents in isolation it is necessary to block inward currents and outward potassium currents with a pharmacological cocktail of channel blockers.

Hippocampal pyramidal cells contain a large voltage-sensitive chloride current (I_{ClV}) that is turned off by depolarisation. The current is active at resting potential and can be blocked by activation of protein kinase c by phorbol esters (Madison *et al.*, 1986). I_{ClV} is blocked by Cd^{2+} (100uM), though this current is not dependent upon Ca^{2+} influx and persists in a Mg^{2+}, Co^{2+} or Ca^{2+}-free EGTA saline (Selyanko, 1984; Madison *et al.*, 1986).

When Cl^- loaded *Aplysia* neurones are hyperpolarised under

voltage-clamp for 30 seconds a slowly developing inward current appears which is carried by Cl^- leaving the cell (Chesnoy-Marchais, 1983). A maximum single channel conductance of 10-15pS has been measured with a half sub-conductance level (Chesnoy-Marchais and Evans, 1986). The hippocampal pyramidal cell chloride current differs from the *Aplysia* current in that it does not require intracellular Cl^- loading in order to be detected.

The electric marine ray *Torpedo californica* is able to stun prey with electrical current delivered from its electroplax organ. Histologically, the electroplax is modified skeletal muscle tissue rich in ACh (nicotinic) receptors. In addition, studies with reconstituted membrane vesicles inserted into planar lipid bilayers have demonstrated a voltage-dependent Cl-selective channel (White and Miller, 1979; Miller and White, 1980). The channels are blocked asymmetrically by stilbene derivatives e.g. 4-acetamido-4-isothiocyanostilbene-2,2'-disulphonic acid (SITS) such that the cis orientation (the side of the bilayer to which membrane vesicles are added) is susceptible to blockade (White and Miller, 1979). In *Torpedo*, single channel conductance is 30pS and the open state consists of rapid excursions into three well defined sub-conductance states; U (20pS), M (10pS) and D (0pS). The rapid zero conductance state is distinct from the long term closed state. The probability of observing the individual states is voltage-dependent. Positive potentials favour the U state, whereas more negative potentials favour M and D states at the expense of the U state (Miller, 1982; Hanke and Miller, 1983).

Both *Aplysia* and *Torpedo* Cl^- channels are thought to exist as dimeric channels with a pair of protochannels which gate independently on a time course of milliseconds but are coupled by a

slower gating process giving rise to 3 conductance states; closed, half-open and open (Chesnoy-Marchais and Evans, 1986).

In skeletal muscles about 65-85% of the resting membrane conductance is due to chloride ions (Hogkin and Horowicz, 1959). This large resting Cl^- conductance stabilises the resting membrane potential. Blatz and Magleby (1983; 1985) have observed three Cl^- selective channels in cultured rat muscle cells. The Cl^- channel with the largest conductance (430pS in symmetrical 143mM KCl) is closed at the resting membrane potential and thus does not contribute to the resting conductance but is activated by depolarisations up to 0mV. The other two channels, however, have smaller conductances and quite different kinetic properties. One channel has fast kinetics, closing within 1ms after opening, while the other has slower kinetics closing within 10ms after opening. The Cl^- with the fast kinetics has been shown to be active at the resting potential and contributes to the resting conductance (Blatz and Magleby, 1985; 1986b). The fast channel has three modes of activity: inactivated, buzz and normal. In the normal mode the channel enters two open and five closed states. The buzz mode occurs less frequently and is characterised by a pair of brief duration open and shut states. Even less frequently, the channel can also enter the inactivated mode where it can close for extended periods of time i.e. seconds to minutes. During the three possible open states the channel conductance is similar (Blatz and Magleby, 1986b).

In summary, I_{ClV} channels show heterogeneity in that they can be activated by hyperpolarising or depolarising command steps depending on the preparation. Some channels may exhibit several sub-conductance states which may themselves be voltage-dependent.

1.242 Calcium-dependent (I_{ClCa}).

In addition to the calcium-dependent potassium permeability first described in erythrocytes by Gårdos (1958), calcium-dependent chloride currents have been described in a number of preparations e.g. *Xenopus* oocytes (Barish, 1983; Miledi and Parker, 1984), rodent lacrimal acinar cells (Marty, Tan and Trautmann, 1984; Findlay and Petersen, 1985) and cultured rat sensory neurones (Mayer, 1985). Depolarisation triggers Ca^{2+} entry which activates the chloride channel to bring about increased permeability to Cl^-.

The surface membrane of mature ovarian oocytes of *Xenopus laeris* possess a transient outward chloride current, i.e. a Cl^- influx, during depolarisations from -70 or -80mV to potentials more positive than -20mV (Barish, 1983). The current is outward during depolarisation because activation occurs at potentials more positive than the chloride equilibrium potential. Anion replacement of the external Cl^- with the impermeant methanesuphonate reversed the outward current to an inward current. The reversal potential for the current gave a predicted Nernstian shift for a change in external Cl^-. Increasing the external Ca^{2+} concentration increased the transient outward current, while Mg^{2+}, Sr^{2+} or Ba^{2+} abolished the current. Thus the transient outward Cl^- current was dependent upon Ca^{2+} influx and was termed I_{ClCa} (Barish, 1983). Furthermore, intracellular injection of EGTA reduced or abolished the current, whereas intracellular injection of Ca^{2+} evoked large outward currents which reversed close to the chloride equilibrium potential (Miledi and Parker, 1984).

Whole-cell voltage-clamp recordings of rat lacrimal acinar cells revealed two types of current induced by carbamylcholine (Marty, Tan and Trautmann, 1984). At low stimulation levels the I_{KCa} (BK channel) was selectively activated. More intense stimulation or

larger doses of carbamylcholine (> 1μM) produced an inward current at the resting potential. This response is mediated by muscarinic receptors. An ACh-evoked current with similar characteristics has been reported in mouse lacrimal acinar cells (Findlay and Petersen, 1985). The inward current was identified as Cl^- ions flowing through selective channels. This Cl current is inward because the equilibrium potential for Cl^- ions is more positive than the resting potential and therefore Cl^- ions flow out of the cell during depolarisations. Noise analysis showed an unusually small single channel conductance of 1-2pS (symmetrical NaCl solutions) with a mean channel open time of 100-250ms. Raising the external Ca^{2+} concentration or using the Ca^{2+} ionophore A23187 evoked an inward current (Marty, Tan and Trautmann, 1984). High intracellular EGTA abolished the ACh-evoked I_{ClCa} in the mouse preparation (Findlay and Petersen, 1985).

Dichter and Fischbach (1977) reported that cultured chick dorsal root ganglion cells (DRG) produced action potentials followed by long-lasting depolarisation which was associated with a conductance increase. Later voltage-clamp studies by Dunlap and Fischbach (1981) identified a persistent inward tail current which was thought to underly the after-depolarisation. Mayer (1985) reported that a depolarising voltage pulse activated an inward current followed by a slowly decaying inward tail current in a subpopulation of cultured rat sensory neurones. The slow inward tail current reversed around -10mV in normal saline and was reversibly blocked by Cd^{2+}, Co^{2+} or Ca^{2+}-free saline. Reducing the external Cl^- increased the current amplitude. The activation kinetics of I_{ClCa} obey a single exponential function with a time constant of 100ms at -10 to 8mV whereas short duration pulses (<60ms) produced a single exponential

decay which hastened upon hyperpolarisation and had a time constant of 200ms at -60mV.

In summary, I_{ClCa} is activated by Ca influx during depolarisation. This calcium-dependency of the chloride current, like that of I_{KCa}, renders it suscseptible to procedures which affect the inward calcium current. Either competatively blocking Ca binding sites with external application of certain divalent cations or chelating intracellular Ca^{2+} abolishes the current. Increasing the external Ca^{2+} concentration or application of the Ca ionophore A23187 enhances I_{ClCa}.

1.243 Proton-dependent chloride current (I_{ClH}).

When exposed to external acidic saline (pH 5.5 or lower), motoneurones from the jellyfish, *Cyanea capillata*, undergo a transient depolarisation associated with a conductance increase (Anderson and McKay, 1985). The amplitude and polarity of this response depends upon the resting membrane potential. At the resting potential of -60mV a typical pH-evoked depolarisation of 30-40mV was observed. The reversal potential lies between -20 and -30mV i.e. around E_{Cl}, shifts +44mV for a 10-fold change in external Cl^- concentration and is unaffected by Na^+ and K^+ manipulations. Furthermore, application of SITS reversibly abolished the pH-evoked response. This evidence indicates a proton-dependent chloride current present in jellyfish motoneurones.

Proton-dependent chloride currents have also been reported in muscle fibres of amphibia (Loo, McLarnon and Vaughan, 1980), modified muscle electroplaque (Hanke and Miller, 1983) and cultured mammalian neurones (Groul, Barker, Huang, MacDonald and Smith, 1980).

1.3 INDUCED EXCITABILITY.

1.31 Introduction.

The electrogenicity of a neurone is not fixed. Several workers have reported that the electrical properties of excitable membranes can be enhanced under different experimental conditions. These changes, however, are not identical.

The experimental manipulations can be subdivided into acute and chronic procedures. Acute procedures involve the isolated preparation and enhance excitability by altering the balance of the existing membrane properties. For example, certain drugs are known to preferentially block outward current channels and these can be used to unmask inward currents flowing through other unaffected channels. Intracellular ionophoresis of chelating agents can be employed to block ion-gated channels and increase ion gradients across the membrane thereby enhancing existing currents.

Chronic procedures involve the intact animal and require the administration of a drug or surgery with an appropriate recovery time. During this period the cell's metabolism is changing in response to structural or metabolic insult and the electrical properties of the membrane are consequently altered.

Experimental changes in membrane excitability have been observed in vertebrates and invertebrates, some examples of which will be discussed in the forthcoming section.

1.32 ACUTE CHANGES IN EXCITABILITY.

1.321 Channel Blockade.

Under normal physiological conditions most insect motoneurone cell bodies are not invaded by action potentials (Pitman, Tweedle and Cohen, 1972: Hoyle and Burrows, 1973: Gwilliam and Burrows, 1980). Slow-graded membrane potential fluctuations in the order of 7mV have been recorded in the soma of the fast coxal depressor motoneurone (termed cell 28 by Cohen and Jacklet (1967)) of the cockroach in response to axonal stimulation. This reflects the antidromic electrotonic spread of action potentials generated at distance from the soma (Pitman, Tweedle and Cohen, 1972). When current intensities of 10nA or more were injected through one of two microelectrodes, the membrane produced a series of dampened oscillations, the amplitude of which increased with applied current (Pitman, 1979). These oscillations may be indicative of a rapid inactivation mechanism for ions which carry the inward current together with a relatively large rise in a delayed outward current (Pitman, 1979).

TEA^+ was originally demonstrated to block the delayed potassium conductance, I_K, in squid giant axon (Tasaki and Hagiwara, 1957) and later shown to block I_K in cockroach giant axons of the ventral nerve cord (Pichon, 1969). TEA^+ (50mM) had previously been reported to unmask Ca-dependent action potentials in crustacean muscle (Fatt and Katz, 1953; Fatt and Ginsborg, 1958). External application of TEA^+ to other insect preparations after 10 - 20mins. produced all-or-nothing action potentials upon intracellular depolarisation. Action potentials of 8 - 20ms duration have been observed in the locust (Goodman and Heitler, 1979) and up to 100ms duration in the cockroach (Pitman, 1979). In the locust FETi motoneurone soma, however, TEA^+-induced action potentials were reversibly abolished by Na-free saline, TTX (10nM), and Co^{2+} saline (10-30mM). These results

demonstrate the existence of mono- and divalent cation channels in the normal somal membrane of this locust neurone. This contrasts with the cockroach preparation where TEA^+ evoked Ca^{2+}-dependent action potentials in which no Na component was detectable (Pitman, 1979).

The aminopyridines have previously been reported to block an early transient potassium current in molluscan neurones (Thompson, 1977). In the locust FETi, application of 3-aminopyridine (3-AP) (2mM) evoked overshooting action potentials and appeared to affect some of the potassium current underlying the resting potential. This was observed as a slow depolarisation leading to spontaneous firing of the cell (Goodman and Heitler, 1979).

1.322 Ion Gradient Manipulation.

Intracellular injection of citrate ions and EGTA also produced all-or-nothing action potentials in the soma of cell 28 (Pitman, 1979). In the cockroach the intracellular citrate, like externally applied TEA^+, produced action potentials which were unaffected by Na-free saline or TTX (4μM) but were reversibly abolished in saline containing Mn^{2+} (40mM). This suggests an inward calcium current which enters the cell via existing divalent cation channels (Pitman, 1979). Citrate ions and EGTA presumably exert their effects by chelating internal calcium ions. This will increase the calcium ion concentration gradient and prevent activation of any calcium-dependent outward current. The effect that citrate ions and EGTA have upon membrane currents depends upon the extent to which they lower intracellular calcium concentration. Increasing the concentration gradient alone was insufficient to produce a calcium spike because increasing the external calcium concentration did not induce a calcium spike (Pitman, 1979).

1.33 CHRONIC CHANGES IN EXCITABILITY.

1.331 AXOTOMY AND COLCHICINE TREATMENT.

1.3311 Cat spinal motoneurones.

Axotomised cat spinal motoneurones undergo classical chromatolytic histological changes which are accompanied by alterations in their electrical properties (Eccles, Libet and Young, 1958; Kuno & Llinás, 1970a; 1970b). Recording from the cell body between 7 and 20 days post-axotomy, spike-like responses of up to 10mV were superimposed on monosynaptic epsps. Spike initiation did not depend upon the amplitude of the underlying epsps and could not be blocked by hyperpolarising the neurone somata. It was concluded that these partial spikes were all-or-nothing events, occurring at discrete areas on the dendrites, which served to increase the synaptic efficacy of the neurones undergoing chromatolysis (Kuno & Llinás, 1970a).

Cat motoneurones projecting to mainly fast-twitch medial gastronemius muscles possess quantitatively different passive electrical properties from those of motoneurones innervating the slow-twitch soleus muscle. Following axotomy these distinctions disappear as the fast (type F) motoneurone appears to de-differentiate and become like the slow (type S) motoneurone (Gustafsson and Pinter, 1984; Kuno, Miyata and Múñoz-Martinez, 1974). In comparison, the input resistance of type S motoneurones was not significantly changed following axotomy. The electrical changes were not observed after lumbosacral dorsal root section, which suggests that the changes are a direct result of axotomy rather than sensory deprivation (Kuno, Miyata and Múñoz-Martinez, 1974).

Further studies on cat spinal motoneurones have shown that the normal activity of the innervated musculature is important for the maintenance of the normal functioning of motoneurones (Czéh, Gallego,

Kudo and Kuno, 1978). Thoracic cord transection or conduction block of the soleus nerve by a TTX cuff after 8 days, caused a significant decrease in after-hyperpolarisation (AHP) of the soleus motoneurones. These changes were prevented by daily stimulation of the sciatic nerve or the soleus nerve more peripheral to the conduction block. It was suggested that normally innervated muscle may release trophic signals which are retrogradely carried by the motoneurones to the cell body and are essential for the normal electrical funtioning of the cell (Czéh, Gallego, Kudo and Kuno, 1978).

The effects of muscle immobilisation upon soleus motoneurones was studied by Gallego, Kuno, Núñez and Snider, (1979). Two weeks after the soleus muscle was immobilised in a shortened position the soleus motoneurones showed a significant decrease in the duration of AHP. If, however, the soleus muscle was maintained in a lengthened position no significant neuronal changes occurred. Furthermore, the decrease in AHP after spinal cord transection (Czeh *et al.*, 1978) could also be significantly prevented by immobilising the muscle in the lengthened position. Gallego *et al.* (1979) concluded that the primary factor responsible for the maintenance of certain motoneurone properties is the metabolic status of the innervated muscle.

1.3312 Insect Neurones.

Axotomy has also been shown to increase excitability in insect CNS neurones which are normally inexcitable. This was first demonstrated in the cockroach, with cell 28 (Pitman, Tweedle and Cohen, 1972), and later in the locust with the fast extensor tibiae (FETi) motoneurone (Goodman and Heitler, 1979). Four to five days following nerve section, the soma of both these neurones produce action potentials which frequently overshoot zero.

Saline substitution with both preparations indicated that the inward current was predominantly carried by sodium (Goodman and Heitler, 1979; Pitman, Tweedle and Cohen, 1972). In the locust preparation, it was demonstrated that although sodium was the major inward current carrier in normal saline, calcium was necessary for the soma spike when the external sodium concentration was reduced by 50% (Goodman and Heitler, 1979).

From the experiments involving acutely induced excitability (see Section 1.321), the soma of the locust FETi has Na and Ca inward current carrying channels and potassium channels which were susceptible to 4-AP and TEA^+ (Goodman and Heitler, 1979) whereas, in the cockroach, the soma of cell 28 has only Ca channels and potassium channels which were susceptible to TEA^+ alone (Pitman, 1979). With both preparations axotomy induced action potentials which were largely Na-dependent, had a shorter duration (typically less than 10ms) and a larger after potential than the action potential induced by TEA^+.

A particular group of neurones situated along the dorsal midline surface of insect thoracic ganglia have somata which are capable of producing overshooting action potentials. These neurones are called dorsal unpaired medial (DUM) cells (Hoyle, Dagan, Moberly and Colquhoun, 1974). The largest of these DUM cells (DUMETi) which

innervates the extensor tibiae (ETi) muscle, was studied in the locust (Goodman and Heitler, 1979). It was established that, under normal circumstances, the DUMETi soma also has inward Na and Ca channels responsible for the soma spike. Following axotomy an increase in neuropile excitability was observed and external Na alone could support the soma spike. The intrinsic differences in excitability of the non-spiking FETi and the spiking DUMETi may reflect quantitative differences in potassium channels (Goodman and Heitler, 1979). If this were the case potassium channels may play a major role in determining membrane excitability.

Colchicine has been found to disrupt axoplasmic flow (Dahlström, 1968) and, in insects, to induce perinuclear ribonucleic acid accumulation (Pitman, Tweedle and Cohen, 1972) characteristic of axotomised insect neurones (Cohen and Jacklet, 1967). In these respects colchicine may be considered to pharmacologically mimic surgical axotomy. Four days after treatment, colchicine treated animals exhibit a similar increase in excitability to intracellular depolarization (Pitman, Tweedle and Cohen, 1972; Pitman, 1975; Goodman and Heitler, 1979). As for axotomy the major inward current carrying ion in both preparations was shown to be sodium, with some calcium contribution in the locust preparation..

The ionic basis underlying the axotomy and colchicine enhanced excitability was clearly different from that seen with acute treatments indicating that the mechanism of chronically enhanced excitability was not a straightforward suppression of an outward potassium current (Pitman, 1975; 1979; Goodman and Heitler, 1979). The underlying mechanism for axotomy may be an increased inward Na current due to *de novo* synthesis and/or insertion of Na channels into the somal membrane which may have been destined for other regions of

the neurone but are unable to get there because of the severed axon (Weins and Atwood, 1982). The intact axon would normally constitute a "sink" for the newly synthesised channels as they would be continuously transported away from the site of synthesis in the cell body. The theory of *de novo* channel synthesis was supported by the action of the protein synthesis inhibitor cycloheximide in retarding, or preventing, axotomy-induced excitability in crayfish neurones (Kuwada, 1981). Colchicine differs in respect from axotomy in that the anatomy of the axon is maintained and axonal conduction or neuromuscular transmission is not blocked. Both treatments, however, block axoplasmic flow and hence disrupt the transportation of substances to and from the soma. If the mechanism of channel transportation to the periphery involves the cytoskeleton, colchicine treatment would cause an accumulation of channels in the cell body. Inappropriate insertion of Na channels in the cell body is, therefore, likely to take place with axotomy and colchicine treatments.

1.3313 Crayfish Neurones.

Axotomy-induced changes in membrane excitability have also been reported in crayfish CNS neurones (Kuwada and Wine, 1981; Wiens and Atwood, 1982). The normally non-spiking soma of the fast flexor motoneurone and the efferent flexor inhibitor neurones were able to consistently produce overshooting action potentials within 36 hours of axotomy (Kuwada and Wine, 1981; Wiens and Atwood, 1982). The action potentials persisted for two weeks and thereafter declined. The increased excitability was observed in the fast flexors which leave the ganglion via the severed root, while the intact contralateral homologue neurone showed normal decremented passive antidromic potentials (Wiens and Atwood, 1982).

Efferent flexor inhibitor neurones with longer proximal axon stumps took longer to develop action potentials. The action potentials could not be restored by resectioning the proximal stump. Behavioural and histological evidence showed that this decline was not due to the re-innervation of the muscles or reconnection of the proximal and distal axon segments. An important observation was that neuropile recordings showed that processes from flexor inhibitor cells were also able to produce action potentials after axotomy. Intracellular staining showed that this excitability was not accompanied by dendritic sprouting. These axotomy-induced changes are only transient and Kuwada and Wine (1981) demonstrated that a neurone does not have to functionally innervate its target, i.e. receive a trophic factor, in order to maintain its characteristic electrical properties.

Interestingly, in the crayfish preparation axotomy did not induce the same responses in all neurones. The electrogenicity of normally-spiking fast flexor neurones was transiently increased

following axotomy (Kuwada and Wine, 1981; Wiens and Atwood 1982), whereas axotomy did not affect the non-spiking tonically active slow flexor motoneurones or the peripheral inhibitor (Kuwada and Wine, 1981). Some interneurones but not others showed increased soma excitability.

Kuwada (1981) went on to investigate the ionic dependence of the enhanced excitability in the soma of the fast inhibitor (FI) neurone. Isolation of the soma from the rest of the neurone confirmed that the electrical changes had taken place within the somal membrane and were not transmitted from distant loci. The inward current of these soma spikes was reversibly abolished by Na-free and TTX (50-500nM) saline and remained unaffected by Ca-free plus Mn^{2+} saline. These results indicated that the primary inward current carrying ion was sodium. TEA^{+} (50mM) had previously been reported to unmask Ca-dependent action potentials in other preparations (Fatt and Katz, 1953; Fatt and Ginsborg, 1958; Pitman, 1979). The FI neurone TEA^{+}-induced regenerative soma spikes were now Na and Ca-dependent 5-7 days after axotomy.

The metabolic dependence of these induced changes was investigated by first increasing the temperature of acclimated animals from 17° to 27°C. The increased responsiveness was still apparent but the onset occurred 24 hours earlier and declined one week earlier. When these warmed animals were axotomised and then exposed to the protein synthesis inhibitor cycloheximide, the characteristic induced excitability was retarded or prevented. Kuwada (1981) suggested, therefore, that axotomy initiates protein synthesis which underlies the transient increase in voltage-dependent Na and K conductances, perhaps by the *de novo* synthesis of appropriate channels.

1.332 DENERVATION.

1.3221 Frog Slow Muscle Fibres.

Normally innervated slow muscle fibres of the frog were shown to be incapable of producing action potentials (Burke and Ginsborg, 1956). The muscle fibres have end-plates at many points along their length which enables membrane depolarisation by end-plate potentials despite the inability to produce regenerative action potentials. Two weeks after denervation by cutting or crushing the sciatic nerve the muscle fibres acquired the ability to generate action potentials which were irreversibly blocked by TTX, indicating sodium as the main inward current carrying ion. The action potential slowly disappeared between 61 and 110 days following denervation. This corresponded to the reinnervation of the muscle fibres by slow-conductiong motor axons. Several fibres which had not received any innervation also showed a reduced excitability (Miledi, Stefani and Steinbach, 1971; Schmidt and Stefani, 1977).

In view of the dependence of motoneurone properties on the integrity of the target muscle (see section 1.331) there appears to be a two-way dependency between motoneurones and their target muscles.

1.34 ANOXIA AND pH REGULATION.

1.341 Introduction.

Carbon dioxide (CO_2), above ambient partial pressure, is routinely used as an anaesthetic in entomology, and clearly has a profound effect on the nervous system. The anaesthetic effect may be attributed to the lipid solubility of CO_2 (Pauling, 1961). In solution CO_2 readily crosses cell membranes (Jacobs, 1940). Inside the cell the CO_2 reacts with water to produce carbonic acid, which rapidly dissociates to produce hydrogen ions (H^+) and bicarbonate ions (HCO_3^-) as in Fig. 1.341 1. The H_2CO_3-HCO_3^- equilibrium is a highly effective buffering system able to withstand wide variation in proton concentration therefore alterations in extracellular CO_2 will rapidly affect intracellular pH. Many aspects of cell structure and function are influenced by pH, proteins are particularly vulnerable to attack by the highly reactive H^+. Such proteins include metabolic enzymes whose catalytic activity has an optimum pH, or structural components of the cell, e.g. transmembrane ion channels. More recently single protons and deuterium ions have been reported to bind and unbind from a single site on an L-type dihydropyridine-sensitive calcium channel from guinea pig ventricular myocytes (Prod'hom, Pietrobon and Hess, 1987). Consequently the channel conductance is reduced to one third of its original value producing a sub-conductance level rather than causing a complete block. The authors suggest that protonation changes the local surface potential which reduces the cation concentration near the mouth of the channel and thus reduces the flow of ions through the channel.

Biological systems, therefore, need to maintain a constant environmental pH in order to function normally. A failure in pH regulation may cause unusual effects and, in extreme instances, be lethal.

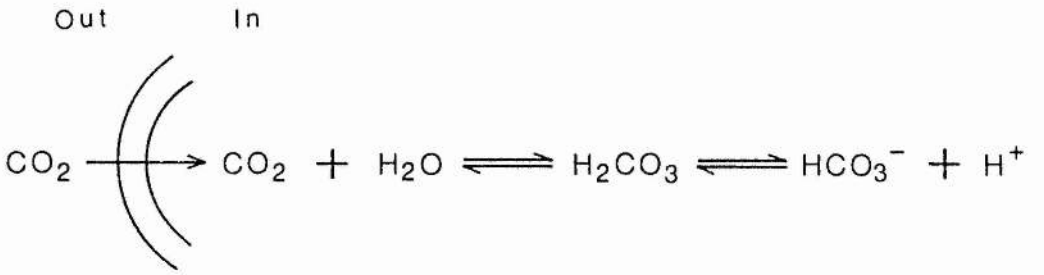


Fig. 1.341 1

Carbon dioxide (CO_2) readily crosses the cell membrane where it reacts with water (H_2O) in the aqueous cytoplasmic environment to produce carbonic acid (H_2CO_3). This reaction is catalysed by the enzyme carbonic anhydrase. Carbonic acid further dissociates to yield bicarbonate (HCO_3^-) and hydrogen ions (H^+).

1.342 The Effect of Anoxia upon Insects.

In locusts, repeated one minute whole-animal exposure to 100% CO_2 causes central neurochemical changes (elevated cephalic octopamine and depression of dopamine) and peripheral neuroendocrine disturbances in juvenile hormone, accompanied by chronic behavioural changes, though it is not clear whether the peripheral changes are a result of central effects (Fuzeau-Braesch and Nicolas, 1981). Few experiments, however, have directly examined the effects of CO_2 upon the membrane properties of insect neurones.

Clark and Eaton (1983) reported that cricket neurones pretreated with CO_2 respond with a slow depolarisation without producing a substantial conductance change. Other treatments which acidified the cell also resulted in a slow depolarisation. In addition, blocking normal acid extrusion with SITS or blocking intracellular pH regulation by removing external chloride ions and thus lowering intracellular chloride ions enhanced the effect of CO_2. Chloride extrusion is involved in a coupled non-electrogenic extrusion of H^+ in some systems (for a review see Thomas, R. C., 1984). The workers concluded that CO_2 exerted its effects by lowering the intracellular pH. Furthermore, the mechanism appeared to be via blockade of a metabolic component underlying the resting potential.

A more recent study by Pitman (1988) shows that between 10 hours and 5 days after whole-animal anoxic exposure (100% CO_2 or N_2) the soma of an identified cockroach motoneurone, cell 28, became capable of supporting Na-dependent action potentials (Pitman, 1988). This observation showed that a fall in intracellular pH brought about by anoxia was not directly responsible for the increase in excitability. Artificially lowering the intracellular pH resulted in a marked depolarisation but was not accompanied by an increase in excitability. Furthermore, histological examination of cell 28 after

anoxia showed that there was no detectable change in RNA distribution which can be seen after axotomy or colchicine treatment (Cohen and Jacklet, 1967; Pitman Tweedle and Cohen, 1972). Since a redistribution in RNA synthesis is thought to reflect an alteration in protein synthesis, it is likely that this change in excitability does not result from *de novo* synthesis of ion channels. The induced excitability brought about by anoxia is therefore hard to reconcile with the axotomy and colchicine-induced excitability. The delay between anoxia and the onset of excitability, however, does suggest a relatively long term change in cellular mechanisms (Pitman, 1988).

For example glycogen storage in insects is also affected by anoxia. In normal cockroaches, glycogen is stored as granules in neural somata at relatively low concentrations. Axotomy or a brief exposure to CO_2 or N_2 induces massive cytoplasmic glycogen aggregates within large somata (Wood, Argiro, Pelikan and Cohen, 1980). The increased glycogen store is thought to be a consequence of stress-induced mobilization and redistribution of sugars derived from body fat (Matthews and Downer, 1973).

1.343 pH AND THE ELECTRICAL PROPERTIES OF CELLS.

1.3431 Crayfish Muscle Fibres.

Under normal conditions crayfish (*Procambarus clarkii*) tonic flexor muscle fibres do not normally generate action potentials. There is, however, a dramatic increase in excitability after prolonged anoxia or treatment with uncouplers of oxidative phosphorylation (Moody, 1978). Application of 2,4-dinitrophenol (DNP) produced all-or-nothing calcium-dependent action potentials after several hours exposure. The observed electrical changes were gradual and a transitory oscillatory response was evident prior to full action potential generation. Two uncouplers were used; carbonyl cyanide-m-chlorphenylhydrazone (CCCP) and *bis*-hydroxycoumarin (dicoumarol). These uncouplers also elicited action potentials.

Three other metabolic inhibitors were employed, each acting on a different mechanism from the previously used compounds. Oligomycin inhibits respiration at the level of ATP synthesis, cyanide inhibits electron transport and iodoacetate inhibits glycolysis. Of these inhibitors only oligomycin evoked action potentials. Pre-treatment with cyanide potentiated the action of dicoumarol whereas pre-treatment with iodoacetate inhibited it.

The mechanism of uncoupler action was thought to be via blockade of mitochondrial calcium buffering and a subsequent elevation in internal calcium (Godfraind, Kawamure, Krnjevic and Pumain, 1971). Moody (1978) argued against this effect for three reasons. Firstly, an increase in internal calcium leads to a decrease in calcium-dependent action potentials in barnacle slow muscle fibres (Hagiwara and Nakajima, 1966); and secondly, an unusual feature of the uncoupler induced action potentials was that they underwent fatigue during repetitive low frequency stimulation. Moody (1978) suggested that intracellular calcium accumulation was responsible for fatigue.

Thirdly the iodoacetate inhibition of the dicoumarol effect implied that the induced excitability was brought about through increased anaerobic glycolysis and thus metabolic acidosis.

The action of the uncouplers is influenced by the existing energy status of the cell. Anoxia (100% nitrogen) can also mimic the effects of the uncouplers and, after 4.5 hours, cause a single fibre to produce repetitive firing in response to a steady depolarising current. Changes in the energy status of the cell brought about by metabolic insult can result in a marked increase in electrical excitability. Moody (1981) suggested that metabolic acidosis affects a membrane conductance already present in the membrane.

To test this hypothesis the intracellular pH of the cell was lowered experimentally (Moody, 1980) using the ammonium rebound technique developed by Aickin and Thomas (1977). The principle involved is prolonged exposure of the muscle to extracellular NH_4Cl which results in intracellular accumulation of NH_4^+. When the NH_4Cl is replaced by normal saline, the NH_4^+ dissociates and diffuses out of the cell as NH_3 leaving behind the free H^+. As the internal pH dropped from 7.25 to 6.4 units the size of the action potential increased until, at pH 6.3 - 6.4, virtually all fibres exhibited all-or-nothing action potentials. A similar response was obtained with CO_2-equilibrated Ringer. The increased excitability was concomitant with a decrease in the delayed rectification. In crayfish muscle low concentrations of externally applied TEA^+ (10-15mM) produced a shift in the I-V relationship similar to that caused by intracellular acidification. Furthermore, low concentrations of TEA^+ were able to unmask calcium spikes. These observations indicate that in crayfish muscle low intracellular pH is able to block the delayed rectifying K conductance which alone is sufficient to unmask action potentials.

1.3432 Ion Conductances.

Using internal cellular perfusion and ion-sensitive microelectrode (Thomas, 1974a) techniques, Byerly and Moody (1986) studied the effects of low intracellular pH upon Ca^{2+} and K^+ conductances in *Lymnaea* neurones. When investigating the outward current the experiments were performed in the presence of external cadmium (0.1mM) in order to block any Ca^{2+}-activated current. Under these conditions there were two remaining potassium conductances, a fast transient current (I_A) and a delayed current (I_K). These two currents were found to be present in different proportions in different cells. I_A was isolated by choosing cells with a strong I_A component and by delivering a conditioning pre-pulse to -90mV to remove inactivation and maximise I_A. Under these conditions low intracellular pH of 5.9 units caused a substantial block of I_A. Interpretation of the effect of low intracellular pH on I_K was complicated due to the contribution of a strong outward proton current, I_H, at potentials more positive than 0mV. The relative contributions of I_K and I_H are, however, different at pH_i 5.9 and 7.3. Under these conditions, and blocking I_K with caesium, the I_H contribution was shown to increase as the pH_i decreased. When I_H was not corrected for, low intracellular pH produceed prolonged currents which were initially interpreted as reduced I_K inactivation. However, once the I_H contribution had been corrected for, lowering the pH from 7.3 to 5.9 units blocked I_K by about 65%. As the internal pH is lowered the H^+ equilibrium potential approaches the K^+ equilibrium potential therefore tail current measurements cannot be used to distinguish these two components. Low intracellular pH has also been found to block I_K in *Helix* neurones (Meech, 1979) and in the squid axon (Wanke, Carbone and Testa, 1979).

Conversley, Umbach (1982), working with *Paramecium*, reported

that there was no effect on the peak amplitude of I_K at low pH_i. The assumption here was that all the outward current was carried by potassium and there was no consideration of another component e.g. H^+ current.

In *Lymnaea* neurones at low pH_i there was also observed a small (1-7nA) outward current at +50mV demonstrated to be carried by K^+. Byerley and Moody (1986) thought this was due to the activation of I_{KCa} by an increased intracellular calcium concentration. Outward H^+ currents also contaminated inward current measurements so these two conductances cannot be readily separated. Estimation of the I_H component exploits the fact that Ca^{2+} currents disappear during prolonged dialysis while H^+ currents remain. Subtraction of measurements taken before and after prolonged dialysis at low pH_i enable the calcium component to be calculated. The calcium current was also decreased at low pH, though there was no distinction as to whether this was due to a direct effect on I_{Ca} or secondary to an internal rise in Ca^{2+}.

In *Paramecium* calcium and barium currents were decreased in low pH_i and enhanced in high pH_i (Umbach, 1982). Intracellular alkylazation caused a reversible increase in early inward currents converting a graded regenerative response to an all-or-nothing calcium spike. These observations indicate that pHi has a direct effect upon the calcium channel in *Paramecium*.

Barnacle muscle cells exposed to CO_2-artificial seawater (ASW) undergo intracellular acidification and exhibit a small increase in Ca_i measured by aequorin light emission (Lea and Ashley, 1978). This was accompanied by increased isometric tension development. Stimulation of Ca^{2+} entry was thought to be involved in this response with some contribution from depolarisation-induced release of Ca^{2+}

from the sarcoplasmic reticulum.

In summary, low intracellular pH reduced I_A, I_K and I_{Ca} in molluscan and squid neurones (Byerley and Moody, 1986; Meech, 1979; Wanke, Carbone, and Testa, 1979). Conversely, I_K was unaffected in *Paramecium* while I_{Ca} was enhanced in *Paramecium* (Umbach, 1982) and barnacle muscle (Lea and Ashley, 1978). By reducing I_{Ca} one might expect I_{KCa} to be reduced, though this was not examined experimentally. Under certain conditions I_H significantly contributes to the net outward current.

1.3433 INTER-RELATIONSHIP BETWEEN Ca_i AND pH_i.

The previous section looked at the effect of pHi and calcium currents across the extracellular membrane. The following section will examine the relationship between calcium and hydrogen ions within the cytoplasm. According to the three mechanisms underlying pH regulation proposed by Thomas R.C. (1984) (see Fig. 1.3442 2), transmembrane Ca^{2+}-H^+ exchange does not occur in the range of preparations so far studied. However, intracellular pH does affect cytoplasmic calcium (Ca_i) in some cells.

Intracellular injection of Ca^{2+} into *Helix* neurones has been reported to cause a fall in pH_i (Meech and Thomas, 1977). The implication was that cytosolic calcium was rapidly accumulated by intracellular organelles in exchange for protons. Pre-treatment with CO_2-saline was previously demonstrated to cause a transient decrease in pH_i, which slowly returned to normal, and also a 3-fold increase in the cells' buffering capacity (Thomas, 1976a). Injection of calcium then produced a greatly reduced change in pH_i. Conversely, in *Helix* neurones intracellular acidification has been reported to reduce internal Ca^{2+} (Alvarez-Leefmans, Rink and Tsien, 1981). Clearly the inter-relationship between Ca_i and pH_i differs between cells and cannot be confidently predicted.

Ahmed and Connor (1980) used intracellular dyes in molluscan neurones to measure pH and $Ca^{2+}{}_i$ changes during electrical activity. They found that voltage-clamp pulses, which induced calcium entry, were followed by internal acidification, detected by a drop in absorbance. In addition, trains of action potentials also produced internal acidification. The effect of pH_i on $Ca^{2+}{}_i$ may not indicate the action of low pH_i on Ca^{2+}-dependent systems. For example, if H^+ competes with Ca^{2+} at Ca^{2+}-binding sites, at low pH_i, Ca^{2+} binding may be reduced despite an increase in $Ca^{2+}{}_i$ (see Moody, 1984).

1.344 pH REGULATION IN SNAIL NEURONES.

Clearly there must be one or more mechanisms which underly pH regulation in excitable cells. The first real advance in this field came with the development of pH-sensitive glass microelectrodes (Thomas, 1974a). These microelectrodes had a small tip diameter of 1μM and enabled the accurate measurement of intracellular pH, initially within snail neurones. Thomas (1974a) measured the average internal pH in *Helix aspersa* neurones and found that the intracellular pH is maintained at a more alkaline level i.e. 0.22 units higher than the expected value if H^+ were passively distributed across the membrane. This suggested that there was a mechanism for H^+ extrusion against its concentration gradient.

The intracellular pH of a normal snail neurone was little affected by manipulating the cell membrane potential, extracellular pH or altering extracellular K^+ and Na^+ concentration.

If the proton extrusion mechanism is metabolically driven, metabolic inhibitors will reduce the available energy and slow down or block the process. Thomas (1974a) reported that anoxia (N_2 exposure), azide and dinitrophenol (DNP) exposure caused a slow fall in intracellular pH.

In solution, external application of ammonia readily crosses cell membranes (Jacobs, 1940). In an aqueous environment ammonia will react with free H^+ to produce ammonium ions (NH_4^+) and hydroxyl ions (OH^-) (Fig. 1.344 1). With snail neurones external ammonia caused a rapid increase in pH. The magnitude of increase was dependent upon the external ammonia concentration. According to the equilibrium equation in Fig. 1.344 1, high external ammonia will drive the equilibrium toward the right. Conversely, intracellular injection of NH_4^+ acidified the cell and the equlibrium shifted to produce NH_3 and H^+.

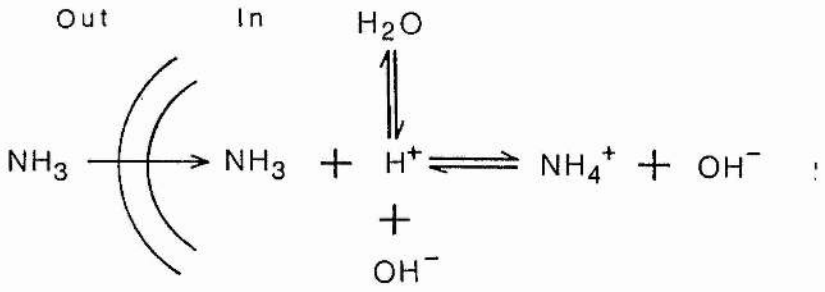


Fig. 1.344 1

Ammonia (NH_3) readily crosses the cell membrane where it reacts with hydrogen ions (H^+) to produce ammonium (NH_4^+) and hydroxyl (OH^-)ions.

External CO_2-Ringer produced a rapid fall in intracellular pH of 0.6 units which returned to normal on replacement with normal Ringer saline. Later experiments (Thomas, 1976a) showed that prolonged CO_2 exposure elicited a rapid, transient fall in internal pH which exponentially returned to normal levels. This further suggested an active process involved in pH regulation as extrusion of H^+ or uptake of OH^- or HCO_3^- would be against their electrochemical gradients (Thomas, 1976a). This initial rapid fall in pH was slowly offset in the presence of bicarbonate ions in the external solution. On return to normal Ringer the internal pH increased and overshot the resting level before returning to normal. Bicarbonate ions were presumed to cross the cell membrane and add to the intracellular bicarbonate levels. According to the equilibrium in Fig. 1.344 1, this would decrease the available H^+ concentration. To test this hypothesis bicarbonate was injected into the cell which responded with a large slow increase in internal pH (Thomas,1974b).

The rapid intracellular response of snail neurones to addition or removal of CO_2 suggests the involvement of the enzyme carbonic anhydrase which catalyses the hydration of CO_2 and the dehydration of H_2CO_3. This view is supported by the observation that the carbonic anhydrase inhibitor, acetazolamide slows the pH response to addition and removal of external CO_2 (Thomas, 1976a).

Intracellular increase of CO_2 will increase the buffering power of HCO_3^- according to the equilibrium in Fig. 1.341 1. Intracellular injection of HCl during CO_2 exposure demonstrated an increased buffering power three times that of normal cells. Once again acetazolamide reduced the effective buffering power of a CO_2-acid loaded cell to injected acid indicating a functional role for carbonic anhydrase (Thomas, 1976a).

1.3441 PROTON-PUMP MECHANISM IN SNAIL NEURONES.

Thomas then focussed on the underlying mechanism of neuronal H^+ extrusion under acid load and, in particular, the role of bicarbonate, chloride and sodium ions (Thomas, 1977). From previous work, external bicarbonate ions will offset CO_2-induced intracellular acidification in snail neurones (Thomas, 1974b). Reducing external HCO_3^- greatly slowed the recovery from CO_2 acid load. This breaking effect of HCO_3^- has been reported in squid giant axons (Boron and De Weer, 1976). Reducing the external chloride ion concentration in snail neurones does not inhibit this recovery. If, however, internal Cl^- is reduced this does slow the rate of recovery. As the intracellular pH recovered after CO_2 exposure the intracellular Cl^- concentration, measured with ion sensitive electrodes, correspondingly decreased. These results suggest that HCO_3^- entering the cell is exchanged for intracellular Cl^- leaving the cell (Thomas, 1976b).

Ionic substitution of Na^+ in the external solution greatly inhibited the pH response to acid load. Ion-sensitive electrodes detected a transient increase in intracellular Na^+ during the recovery time. When the Na-K pump was blocked by removing external K^+, the intracellular Na^+ continued to rise until normal Ringer was superfused. It is probable that sodium therefore enters the cell in exchange for protons during pH regulation.

On the basis of these experiments a dual exchange mechanism was postulated involving Cl^--HCO_3^- and Na^+-H^+ systems. Were these two systems linked or independent from one another? To test this hypothesis both systems were blocked. The Na^+-H^+ by removing external Na and the Cl^--HCO_3^- by the stilbene derivative, SITS, which blocks anion exchange. Blockade of both systems did not at once produce an additive inhibition of recovery which suggested that the

mechanisms were linked to the same process. Thomas (1977) proposed a membrane exchange mechanism which is schematically represented in Fig. 1.3441 1. The ionic mechanism involves a coupled non-electrogenic exchange of ions betweeen the interior and exterior of the cell which requires metabolic energy in order to function (Thomas, 1974a).

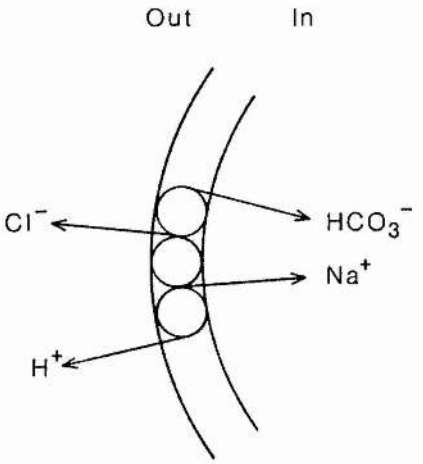


Fig. 1.3441 1

Proposed transmembrane ionic exchange mechanisms underlying pH regulation in snail neurones (adapted from Thomas, 1977).

1.3442 pH REGULATION IN OTHER EXCITABLE CELLS.

Intracellular pH regulation in snail neurones (Thomas, 1974b; 1976a; 1977), squid axons (Boron and Russell, 1983) and barnacle muscle (Boron, McCormick and Roos, 1981) appear to have the same ionic requirements and, by inference, a similar underlying mechanism. Not all invertebrates have the same regulatory mechanism. Moody (1981) reported that crayfish neurones respond in a similar way to snail neurones to acid load i.e. intracellular acidification with a slow recovery. This recovery was dependent upon external Na^+ and HCO_3^-. Removal of HCO_3^- slowed the recovery but did not abolish it whereas removal of Na^+ almost totally blocked the recovery. Application of SITS slowed the rate of recovery to that usually observed in HCO_3^--free Ringer. In addition, SITS eliminated the dependence of the rate of recovery on external HCO_3^- which suggests two independent regulating mechanisms. Fig. 1.3442 1 shows schematically the linked H^+-Na^+ and Cl^--HCO_3^- system and, in addition, a direct H+-Na+ exchange (Moody, 1981).

From the literature it seems that pH regulation in a variety of animal cells can be achieved by a combination of ionic mechanisms (for review see Thomas, 1984). In summary, there are three basic mechanisms shown in Fig. 1.3442 2 as a schematic representation adapted from Thomas (1984). Various preparations employ a combination of systems to regulate intracellular pH.

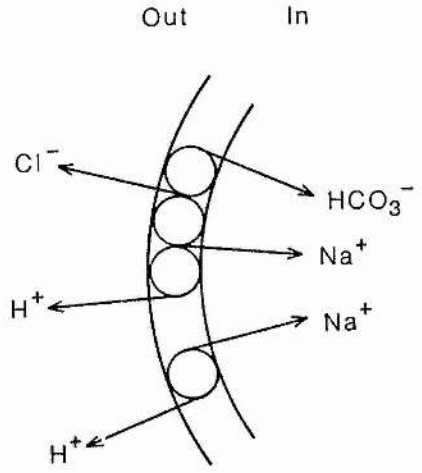


Fig. 1.3442 1

Proposed transmembrane ionic exchange mechanisms underlying pH regulation in crayfish neurones (adapted from Moody, 1981).

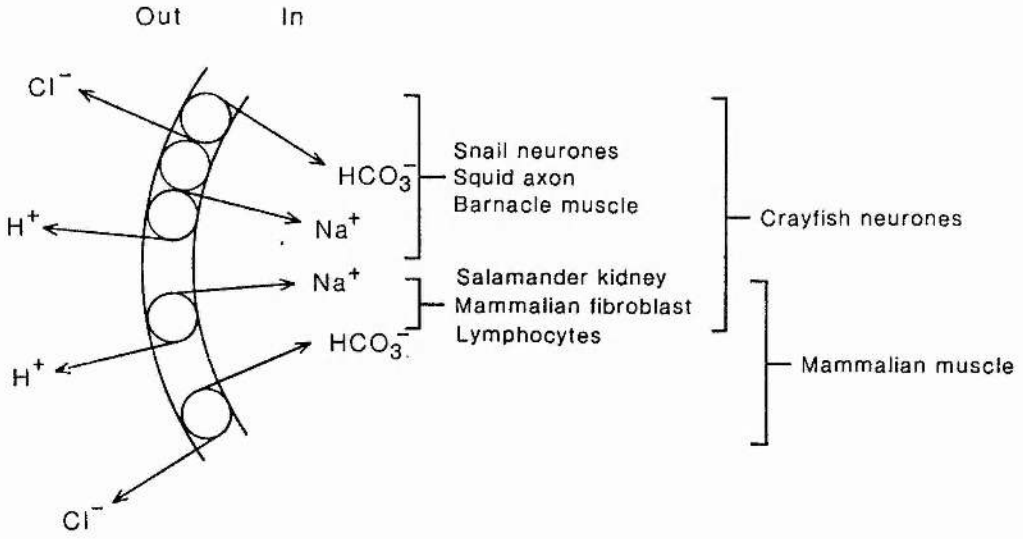


Fig. 1.3442 2

Possible combinations of the three basic mechanisms underlying pH regulation in different animal cells (adapted from Thomas, R.C. 1984).

1.4 THESIS IN CONTEXT.

The group Insecta contain more species than all the rest of the animal kingdom put together. The enormous success of this group has been attributed to their diversification throughout the environment. The consequences of this diversification have not always been benificial to mankind. All over the world pest species destroy vast amounts of foodstuffs causing significant social and economic hardship. In an attempt to control the situation pesticides have been used extensively. This has resulted in reduced effectiveness of these agents because some species develop resistance to them. Although the use of pesticides is not the only way to control insect pests, they are relatively cheap, easily obtainable and offer some protection and will thus continue to be widely used. There is, therefore, an increasing interest shown by the multinational companies in the development of biologically and ecologically safe, effective insecticides.

Most insecticide compounds affect the central nervous system. Surprisingly little is known about the neurobiology of insects. They have been used in the study of the neurobiology of behaviour (Hoyle, 1977) though it was not until the late 1960s that the first intracellular recordings were made from insect CNS (Callec and Boistel, 1967; Kerkut, Pitman and Walker, 1968). Insect pharmacology and neural biophysics are relatively neglected fields which are currently gaining special interest.

Apart from the applied nature of the current research, insect neuroscience offers interesting challenges in its own right. To what extent is the diversity of insects reflected in the organisation of their nervous systems? Do insects possess characteristics which are peculiar to this group and how similar are they to other groups? Thorough pharmacological and biophysical investigations are required

in order to answer these questions.

Most insect motoneurone cell bodies are inexcitable i.e. they can not sustain action potentials but may be enabled to do so under a variety of experimental conditions. In order to more fully understand the basis of this inexcitability and its implications this study has focussed on outward currents and, in particular potassium currents which are most likely to carry a major part of the outward current in these motoneurones. From the literature cited above it may be seen that there are many types and sub-types of potassium channels and associated currents which can provide a range of conditions necessary for the fine adjustment of a cell's excitable status. In context, this thesis is presented as an attempt to understand, in a mechanistic way, the ionic basis of insect neural excitability and the ways in which it can be altered. The aims of this thesis were:

i) To characterise, in terms of classically described outward currents, the macroscopic ionic currents in an identified insect motoneurone of the cockroach, *Periplaneta americana*.

ii) To investigate the neurone's excitability after acute and chronic procedures which are known to affect the electrical characteristics of other insect neurones.

2. METHODS AND MATERIALS.

2.1 ANIMALS.

2.11 Maintenance and Dissection.

A laboratory culture of *Periplaneta americana* was maintained at 27°C in plastic containers. The animals were supplied with water and fed moist dog food twice weekly. When necessary this stock was supplemented with animals obtained from Blades Biological (Kent). All experiments were performed on adult males. For the dissection, animals were beheaded and pinned ventral surface uppermost on a Sylgard dish. The ventral nerve cord was then exposed and dissected out into normal cockroach saline (See APPENDIX I for composition of salines). Dissections were performed under a Kyowa zoom binocular microscope (70X - 45X).

For electrophysiological work the nerve cord was positioned on a small black plastic slide (10 X 250mm) with the metathoracic ganglion dorsal side uppermost on an Araldite ridge (Fig 2.11 1). Both ends of the nerve cord were secured with plastic bands. Once firmly positioned, one or two drops of 4% methylene blue were applied to the ganglion surface in order to visualise the sheath before it was mechanically removed from the anterior portion of the ganglion. The preparation was then immediately transferred to the Perspex experimental chamber.

2.12 Anoxia experiments.

Animals were placed in a sealed container with a moistened cotton wool ball then gassed with 100% carbon dioxide. After two hours exposure to CO_2 the animals were removed and allowed to recover in individual boxes supplied with food and water at 27°C. Between 12 and 24 hours. thereafter, individuals were removed and dissected for electrophysiological investigation as described above.

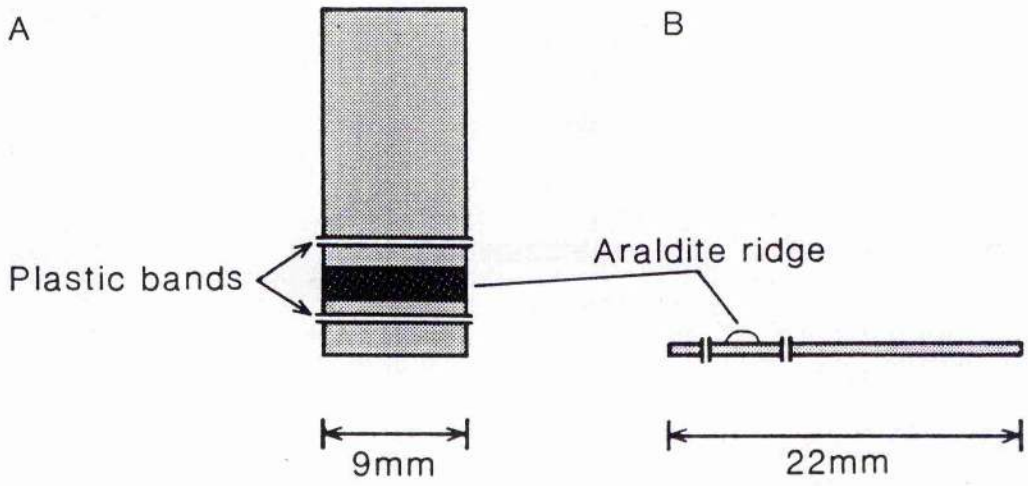


Fig. 2.11 1

The preparation slide showing **A.**) front and **B.**) side views of the ridge over which the nerve cord was placed and secured on either side by plastic bands.

2.13 Axotomy experiments.

Operations were performed under a Kyowa binocular zoom microscope in a Bassaire laminar flow cabinet. Before each operating session the fan was turned on for 15 to 20mins. and the inside of the cabinet and microscope swabbed with ethanol (96%). All dissecting instruments and restraining pins were steeped in ethanol (96%) containing 1% liquid savalon and sterilised in a methylated spirit flame before use.

Animals were lightly anaesthetised with CO_2 and restrained ventral surface uppermost on a Sylgard dish. CO_2 was administered throughout the experiment in order to maintain anaesthesia. A small, unilateral incision was made around the metathoracic basisternum and antecoxite leaving the anterior edges intact. This cuticular flap was then pulled back to expose the tracheal system overlying the metathoracic ganglion. The posterior region of the ganglion was cleared of tracheal tubes, care being taken to minimise disruption. Nerve 4 was then cut 2-3mm distal to the ganglion using micro scissors (John Weiss & Sons Ltd.). Nerve 4 lies slightly anterior and dorsal to the large nerve 5 to which it is connected by a small isthmus near the ganglion. One drop of cockroach saline containing antibiotic (1% Kanamycin) was applied to the wound before the cuticular flap was replaced. A small section of wing-tip was then placed over the wound and sealed around its edge with molten low melting point paraffin wax. Animals were then allowed to recover in individual boxes supplied with food and water at 27°C. Between 4 and 6 days thereafter, animals were killed and their nerve cords removed and prepared for electrophysiological recording.

2.2 ELECTROPHYSIOLOGY.

2.21 Experimental Chamber.

The experimental chamber (Fig. 2.21 1) contained circulating oxygenated sucrose (100 mM) saline (APPENDIX I). Pure oxygen (BOC) was bubbled through a narrow tube positioned in the right hand compartment of the chamber. The rising O_2 bubbles setup an anticlockwise circulation of the saline around the preparation. Saline could be gravity fed, and a surface suction apparatus ensured a constant bath volume of 2ml.

The bath electrodes were connected to the experimental chamber via an Agar bridge. This arrangement enabled the superfusate in the main chamber to be changed without disturbing the electrolytic environment of the bath electrodes. All bath electrodes were made from silver wire (1mm diameter) and beaten into a flat spade-shape (approximately 2mm wide) in order to increase the conducting surface area. Electrodes were electrically coated with chloride using 1M potassium chloride and a 9V battery. This prevented electrical polarisation of the electrodes and thus allowed the recording of stable potentials.

2.22 Intracellular Instrumentation.

Microlectrodes were positioned in the bath using a pair of Prior micromanipulators fitted with reduction drives in two orthoganal horizontal planes. The preparation was illuminated using a beam from a microscope lamp (W. Watson and Sons LTD.) located outside the Faraday cage, which reflected off a mirror inside the cage and focussed down onto the preparation by a movable convergent lens (Fig 2.22 1). Once the preparation was in the bath, a binocular zoom microscope (Nikon) could be swung into position in front of it, allowing the preparation to be viewed horizontally at a magnification range of 18X - 80X.

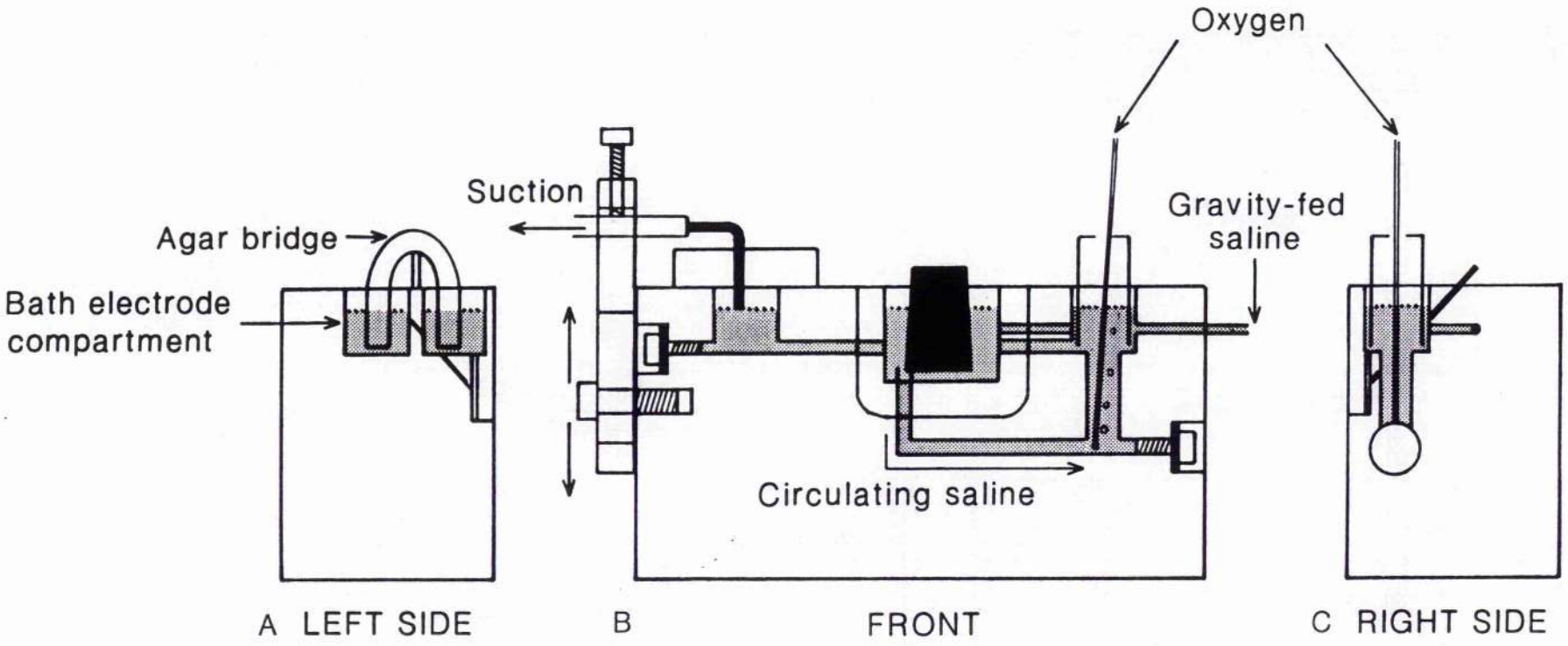


Fig. 2.21 1

The experimental chamber showing A.) left, B.) front and C.) right side views. The stippling indicates areas containing saline. Saline enters the chamber under gravity and oxygen bubbling in the right hand compartment sets up an anti-clockwise circulation around the preparation indicated by the arrow. The saline is maintained at a constant level by the surface suction apparatus which collects the waste in a vacuum trap within the Faraday cage.

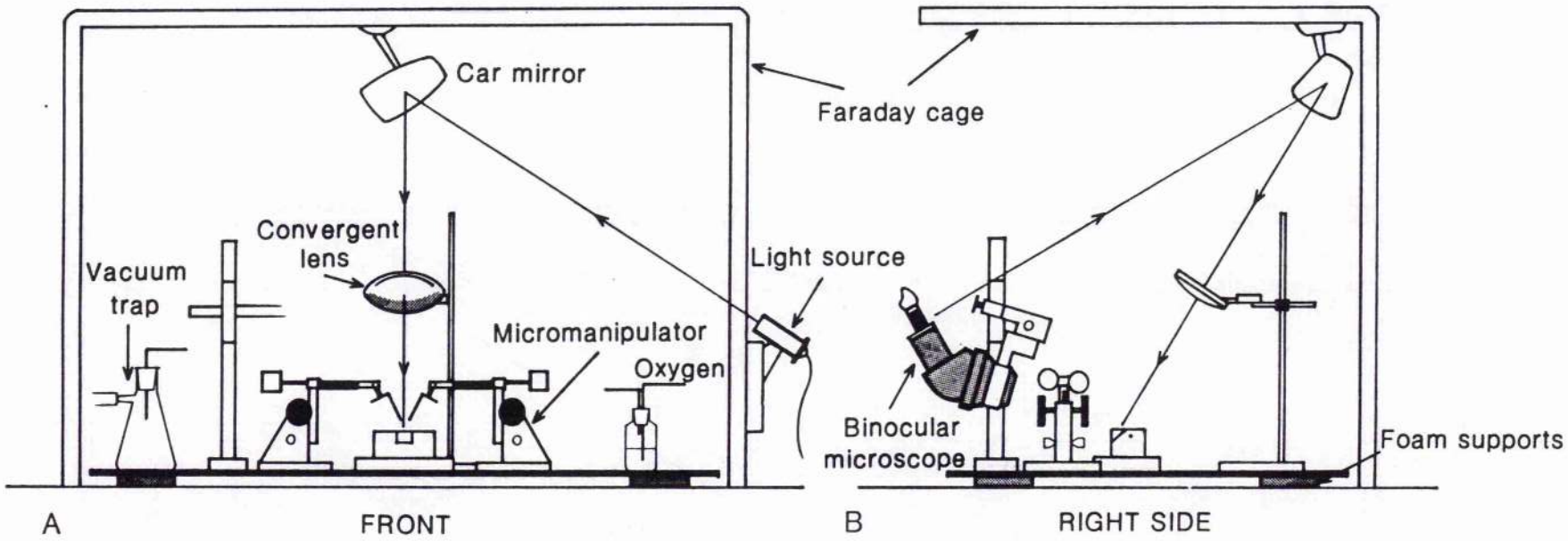


Fig. 2.22 1

The experimental rig showing A.) front and B.) right hand views. Micromanipulators were positioned either side of the experimental chamber. The light source was secured outside the Faraday cage and the beam reflected off a mirror and focussed by a movable convergent lens on to the preparation below. The binocular microscope could then be swung in front of the chamber when required.

Micropipettes were drawn from 1mm internal diameter thin-walled filament glass (Clark Electromedical Instruments) on a Narishige PE-2 vertical electrode puller. The micropipettes were filled with 4M potassium acetate. Recording microelectrodes had a resistance of between 20-25 megohms while the current-passing electrodes were bevelled to a resistance of 7-10 megohms using a laboratory-made beveller.

Both microelectrode holders were directly attached to amplifier headstages which in turn were connected outside the Faraday cage to laboratory-made Intracellular Recording amplifier/bridge systems (APPENDIX IIA). A reed relay in the headstage enabled recording or stimulation via the intracellular microelectrodes.

Once the preparation was positioned in the bath a fine jet of saline was manually directed around the area of cell 3 in order to clear surrounding glial cells. When the rounded profile of the nerve cell body was evident, the microelectrode tips were lowered into the saline and positioned over the cell surface. At this stage an earthed brass shield was carefully placed above the bath between the microelectrodes to reduce capacitative coupling. Before impalement, both microelectrodes were balanced through the bridge circuit. For double electrode experiments the bevelled microelectrode was first lowered onto the cell suface until a superficial depression was evident. Signals were monitored on the oscilloscope and surface contact with the neural membrane was indicated by a small voltage deflection. A succession of -20nA, 10ms duration current pulses were applied to the microelectrode in order to facilitate impalement which was characterised by a sudden negative shift in potential of -65 to -70mV.

All drugs (APPENDIX IIIA) were directly applied to the right

hand compartment of the experimental chamber and all drug concentrations refer to the final bath concentration. Drugs were dissolved in normal saline, with the exception of verapamil which was initially dissolved in distilled water to produce a concentrated stock solution and then further diluted with normal saline.

2.23 Current-clamp.

Under current-clamp the bioelectric signals from amplifier II were displayed on a Dual Beam Tektronix (5103N) storage oscilloscope. Applied current was measured using a virtual earth current monitor of switched gain (APPENDIX IIB) and displayed on the second oscilloscope channel. Current was injected into the cell using the relay circuit in the headstage of the first intracellular recording system (amplifier I) thus bypassing the recording circuit (APPENDIX IIC).

2.24 Voltage-clamp.

The output from amplifier II (recording membrane potential) and a reference bath electrode were fed into a differential amplifier to correct for any potential drop across the bath and agar bridge. The output from the differential amplifier went directly into the voltage-clamp amplifier, designed by M. Thomas (APPENDIX IID). The voltage compliance of this amplifier was +/-100V in order to achieve fast voltage clamps with large command pulses. The output from the voltage-clamp amplifier was fed directly into the current microelectrode, bypassing the recording amplifier (APPENDIX IIE).

2.25 Computer-controlled Experiments.

Command signals applied to the input of the voltage-clamp amplifier were controlled via a UNILAB interface (532.001) and a second interface (designed and built in the laboratory) linked to a BBC B series microcomputer (APPENDIX IIE). From the software a number of experimental command pulse regimes could be employed (see Fig 2.25 1):

A. 25 positive pulses sequentially incrementing by +10mV from the holding potential.

B. 25 pulses positive and negative from the holding potential sequentially incrementing by +/-10mV.

C. A positive going pre-pulse (pulse (I)) immediately followed by a test pulse (pulse (I)) to a more negative potential. The test pulse sequentially incrementing by +10mV.

2.26 Data Capture.

All current- and voltage-clamp data were recorded on FM tape (AMPEX) using a RACAL Store 4DS recorder. Data were subsequently played back and individual responses photographed from the oscilloscope screen using a Practica SLR body with an oscilloscope screen converter (Shackman Instruments Ltd). Kodak Kodalith ortho film type 3 (35mm) was used and developed with Ilford PQ Universal developer according to APPENDIX IV, under a Rattan 1B safelight.

Current measurements from individual responses were obtained by one of two methods. Firstly, for regimes A and B, a menu driven option enabled digitised measurements to be sampled at one or more positions along the current response. Data were then written to disk and later recalled on screen or a hard copy obtained using a data reading program designed for each experimental regime. Secondly, tail current measurements obtained from experimental regime C were taken from the enlarged image of the film negative.

Data from some experiments were played, via the UNILAB interface into a BBC (Series B) microcomputer. This procedure was controlled by the FASTAV (fast signal averaging) program (UNILAB) which enabled either single current traces or averaged responses to be recorded. Data were then written to disk, or a hard copy obtained on a single

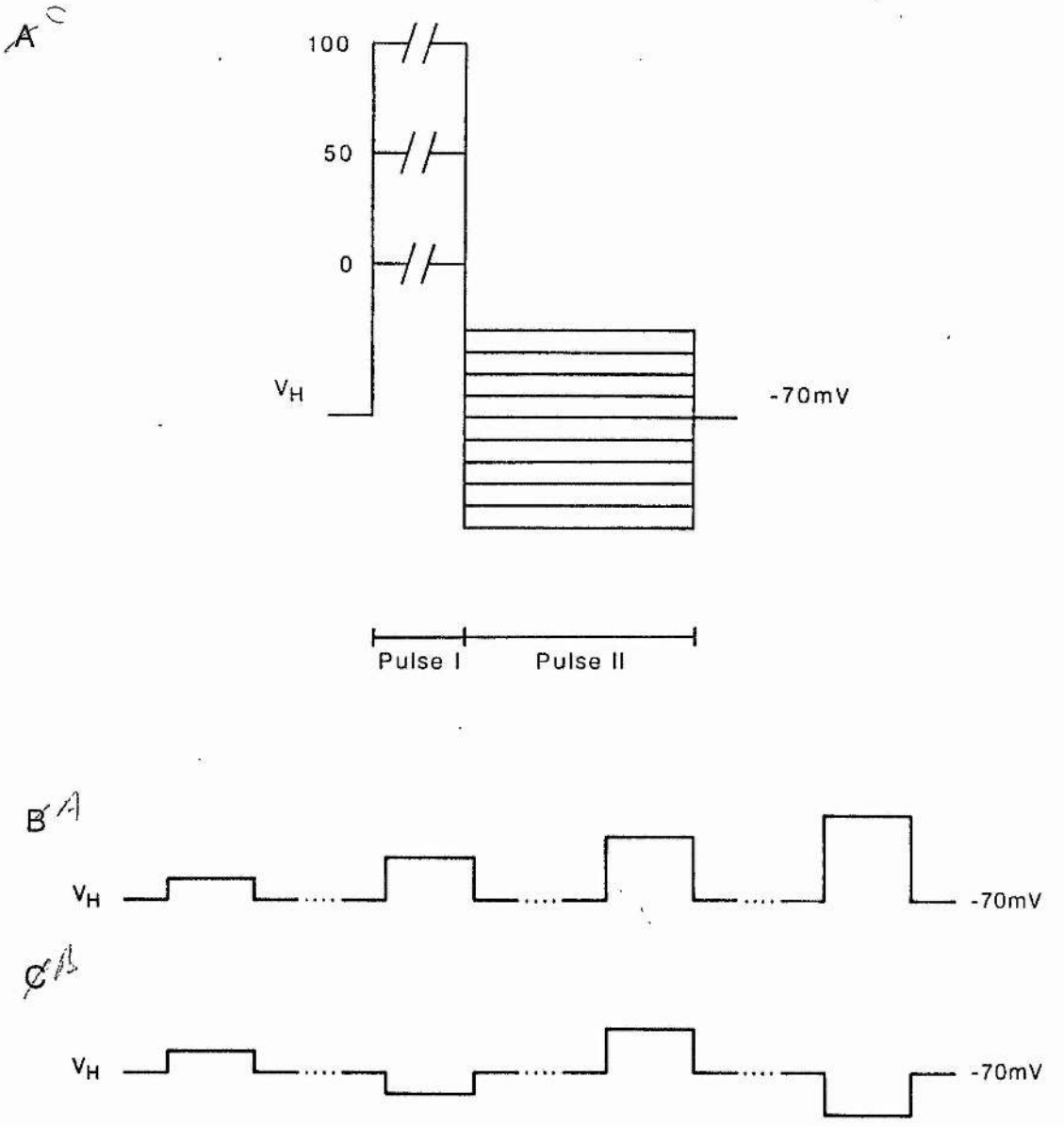


Fig. 2.25 1

Schematic representation of the command pulse regimes: **A.)** 25 steps positive of variable duration successively incrementing by +10mV; **B.)** 25 steps positive and negative of variable duration successively incrementing by +/- 10mV; **C.)** a pre-pulse (Pulse (I)) of variable magnitude and duration immediately followed by a test pulse (Pulse (II)) to a more negative potential which successively incremented by +10mV.

channel chart recorder (JJ Instruments).

2.27 Graphs.

Current-voltage relationships were constructed on a Tandon PCA computer using the Harvard Graphics package (Microsoft). The line graph option was selected which gave a curve of best fit through the data points. Graphs were plotted on a Gould Colorwriter (6120).

2.3 MORPHOLOGY.

2.31 Cobalt Dye Injections.

For intracellular staining, neurones were impaled with a single microelectrode filled with 100mM hexammine cobaltic chloride. Cobalt ions were ionophoretically injected into the cell, for up to 40mins., using +20nA DC generated by a constant current source. Current was delivered directly to the microelectrode using the relay in the pre-amplifier headstage to bypass the recording circuitry. After dye injection the preparation was removed from the bath and the slide then washed in fresh saline for 10mins. before treatment with a dilute solution of ammonium sulphide in saline (1-2 drops of ammonium sulphide in 5ml of saline) for up to 15mins. This was performed in a fume cupboard. The ammonium sulphide reacts with cobalt to produce a black precipitate. At this stage the superficial cell body was frequently visible under a binocular microscope. Ganglia were then washed in fresh saline (10mins.) and transferred to a 1:1 solution of 70% ethanol saline and saline for 10mins. and then finally to 70% ethanol saline. At this stage the preparation could be stored at 4°C indefinitely before moving on to the intensification procedure (APPENDIX V).

After staining had been intensified, the preparation was cleared in histological creosote and mounted in Canada balsam, to allow

detailed examination under a compound microscope. Line drawings were achieved using a projection attachment on a Gillett and Sibert compound microscope. By racking through the focal planes the 3-dimensional branching pattern could be followed and traced onto paper (a 2-dimensional representation).

In order to visualize the 3-dimensional dendritic branch pattern, stereoscopic photographs of whole-mount stained preparations were taken. These were achieved using a Leitz luminar lens with a camera attachment connected to an automatic exposure unit (Vickers Instruments). Recordak AHU Microfilm 5460 (Kodak) was used and processed as in APPENDIX IV. Stereoscopic views were achieved by tilting the adapted specimen stage, in parallel to the camera, 8° toward the left and right. Left and right paired photographs were then mounted adjacently and viewed with binocular glasses.

3. RESULTS.

3.1 MORPHOLOGY.

Figs. 3.1 1A and B show stereoscopic photographs and a corresponding line-drawing of cell 3 (designated by Cohen and Jacklet, 1967) from the metathoracic ganglion of the cockroach *Periplaneta americana*. Cell 3 is bilaterally symmetrical with the cell body nestling between the anterior connective (nerve 1) and nerve 2. A small isthmus from nerve 2 lies across its cell body (personal observation). The cell body is the largest of the neurones in this area (approximately 70-80μM in diameter) and can be easily distinguished. The dendritic tree of cell 3 is confined to the ipsilateral neuropile while its single axon leaves the ganglion via nerve 4. The characteristic morphology of cell 3 allowed unequivocal identification.

Cell 3 is a "fast" motoneurone which innervates the bifunctional basalar/coxal depressor muscle 177C (Carbonell, 1947). During flight this muscle acts upon the thoracic box to cause wing depression (Fourtner and Randall, 1982). During flight the distal part of the 177C is held rigid by tetanic contractions of the femoral flexors, thus facilitating deformation of the thoracic box. During walking the muscle causes extension of the femur (Fourtner and Randall, 1982). The origin (fixed end) of muscle 177C occurs on the episternum and the insertion (moving end) on the trochanter.

During walking cell 3 is a synergist of cell 28 (designated by Cohen and Jacklet (1967) and also termed D_f by Pearson and Iles (1970)), another identified "fast" motoneurone located on the ganglionic ventral surface, which innervates the coxal depressor muscles 177D, 177E, 178 and 179 (Carbonell, 1947; Pearson and Iles, 1970) and causes extension of the coxal-trochanteral joint.

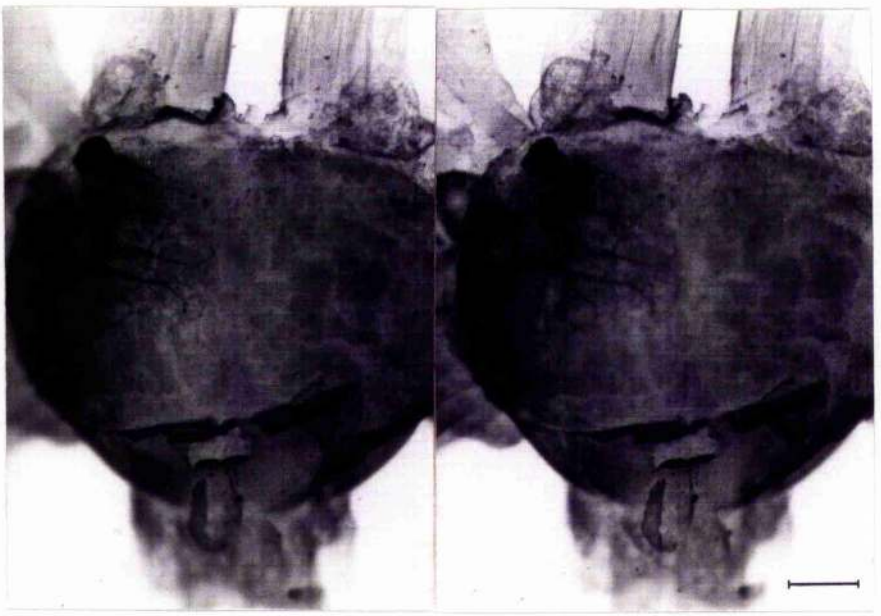
Fig. 3.1 1

Whole mount preparation of a unilateral intracellular cobalt fill of cell 3 within the metathoracic ganglion.

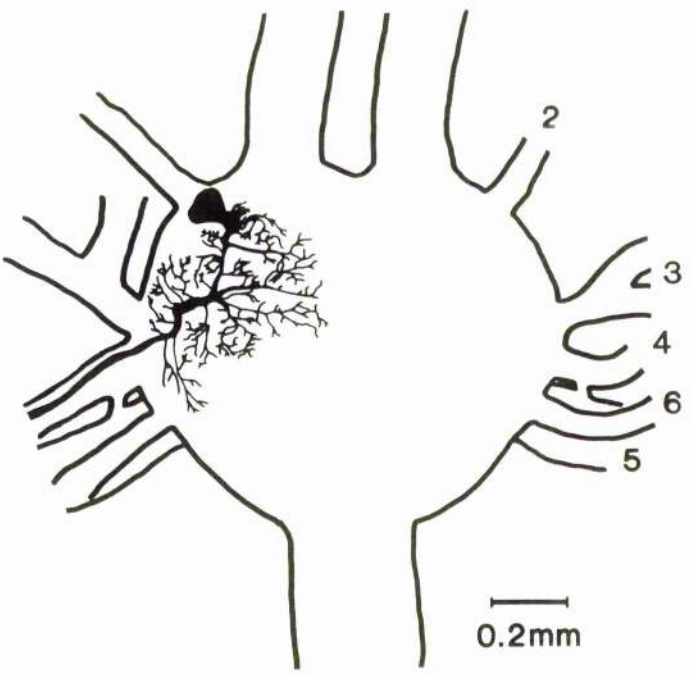
A. Paired stereoscopic views of cell 3 showing the 3-dimensional dendritic branch pattern confined to the ipsilateral neuropile. Scale bar: 0.2 mm.

B. Corresponding line drawing of the same preparation.

A



B



3.2 VOLTAGE-CLAMP

3.21 NET OUTWARD CURRENTS.

3.211 Normal cell response.

Holding the cell at -70mV (near its physiological resting potential of between -70 and -75mV) and successively incrementing the command steps by +10mV produced a net outward current which developed significantly at potentials more positive than -50mV. The maximal current response for short duration command pulses increased in an approximately linear manner as the command step approached +100mV (approximately). Furthermore, as +100mV was approached, a delay in the current rise was evident where the currents laboured to attain a maximal value (arrowhead Fig. 3.211 1Ai). The delay in the current rise began to develop around command potentials of +30mV and can be seen in Fig. 3.211 1B (arrowhead) as a notch in the rising phase of the outward current. As the command potential was increased beyond +90mV the current rise was further delayed until the response dropped to a new, lower level before continuing to rise. This is illustrated in Fig. 3.211 1Ai where selected current traces have been superimposed. The delayed rise was no longer observed after the fall in current response beyond +90mV. Note that the shape of the currents before and after the current delay were different. The former showed some inactivation whereas the latter appeared not to show inactivation and gradually increased for the duration of the command pulse (100ms).

Current measurements taken at 50ms into the command pulse produced an N-shape I-V relationship. In order to standardise the description of forthcoming I-V graphs Fig. 3.211 2A shows a typical I-V curve with the designated regions.

Fig. 3.211 2B shows an I-V relationship from a normal voltage-clamp run without annotation, the outward current developed at

potentials more positive than -50mV. After a slow start the currents increased in a linear manner giving rise to the lower arm of the curve. In this experiment the chord conductance (taken between 0mV and +50mV) was 26μS in normal saline. The hump typically peaked between 3.0 and 4.0μA between command potentials of +80 to +130mV (Fig. 3.211, 2B). The exact position and magnitude of the N-shape varied between preparations. In this experiment the magnitude of the negative conductance region was 1.3uA and had a chord conductance (between +90mV and +100mV) of 100μS in normal saline. After going through the negative conductance region and reaching the trough the currents began to increase once more giving rise to the upper arm of the curve. The increase in currents in the upper arm was not linear and in many experiments was subject to increase particularly after superfusion with saline.

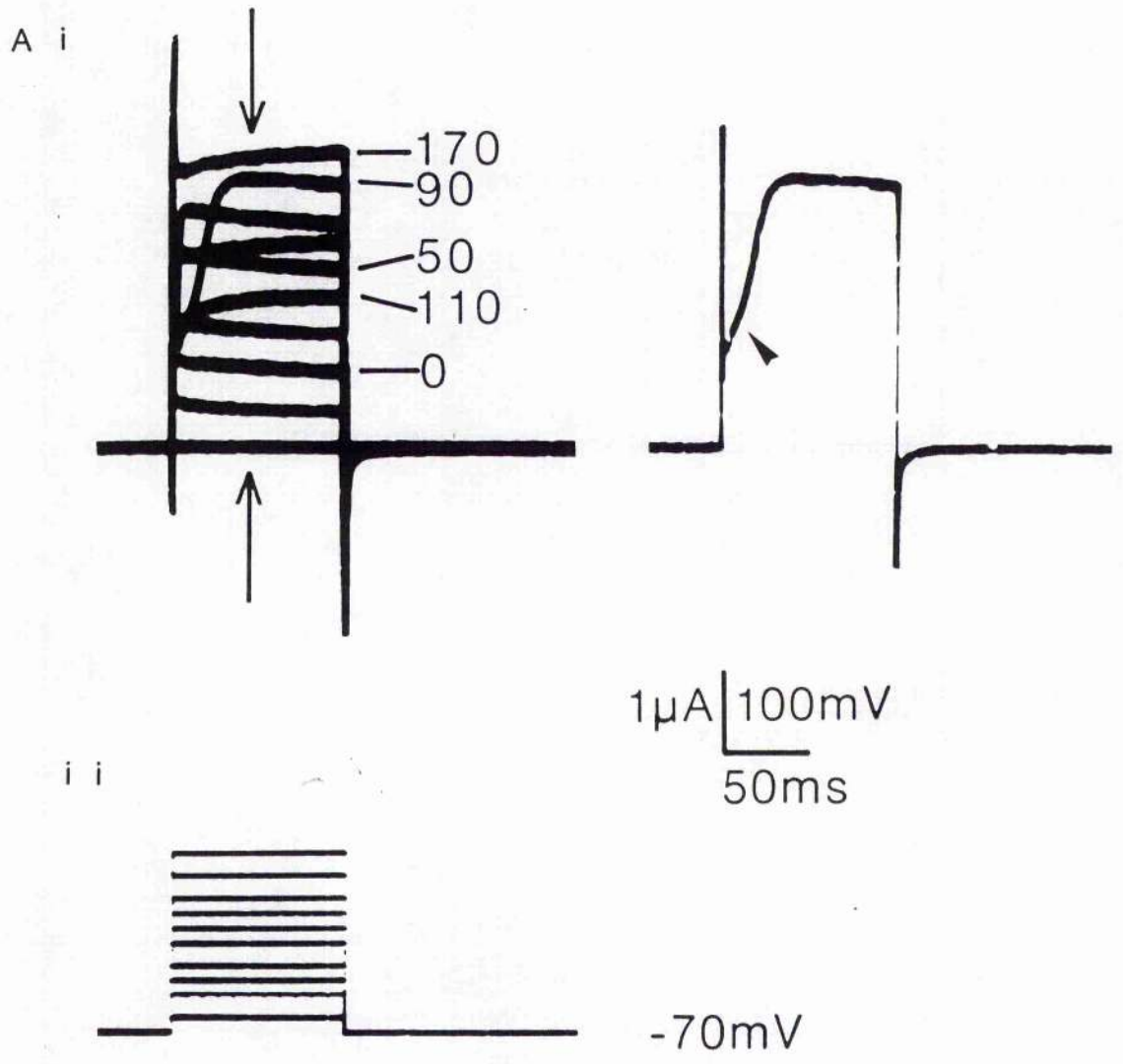


Fig. 3.211 1A

Net outward currents evoked by depolarising command steps.

Command pulse duration 100ms.

Ai. Superimposed net-outward current responses. Selected command potentials shown on the right. Single response to a command potential of +90mV shows the delayed rise time of the outward current (arrowhead). Arrows denote the position of current measurements (50ms) and graphed in 2B.

Aii. Corresponding voltage steps which evoked the currents in Ai.

Holding potential: -70mV.

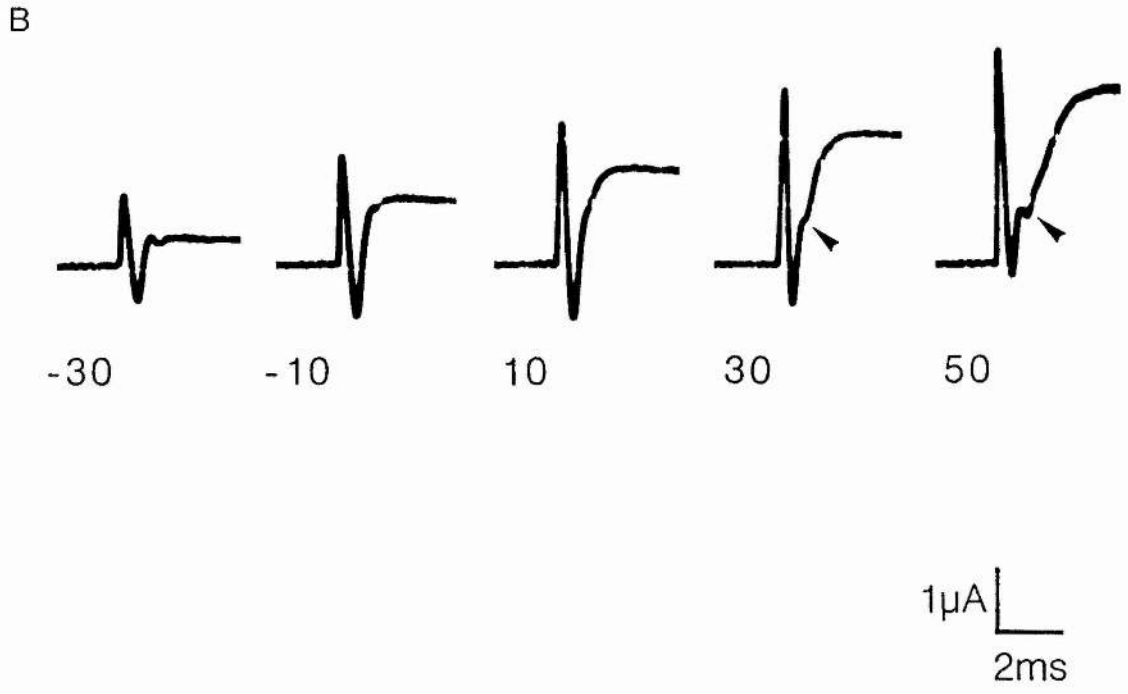


Fig. 3.211 1B

Emergence of the delayed current rise of the net outward currents

Selected current responses taken at command potentials of -30, -10, +10, +30 and +50mV. Expanded time-base of currents shown in Ai. Arrowheads denote the emergence of the delayed rise time around potentials of +20 to +30mV. The delay increased with further depolarisation.

Holding potential: -70mV.

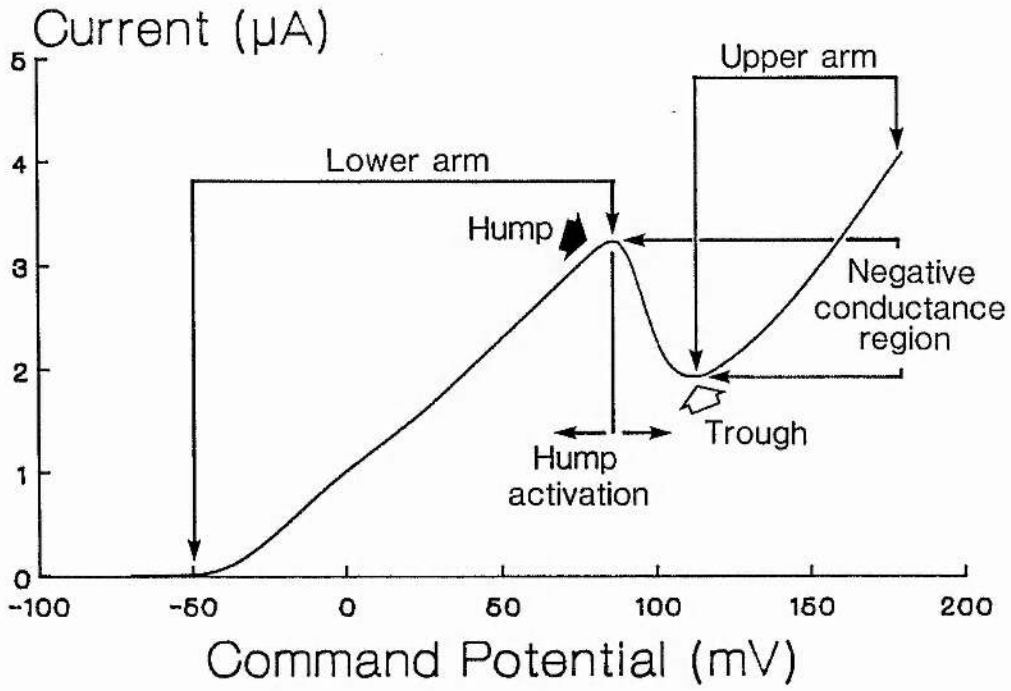


Fig. 3.211 2A

A typical N-shape I-V relationship showing the designated regions of the curve.

The linear increase in currents gives rise to the lower arm of the curve which increases to a maximum called the peak of the hump. The positioning of this hump along the voltage axis is called the hump activation. The current then declines through a negative conductance region toward a trough. Thereafter the current increases in a non-linear manner giving rise to the upper arm of the curve.

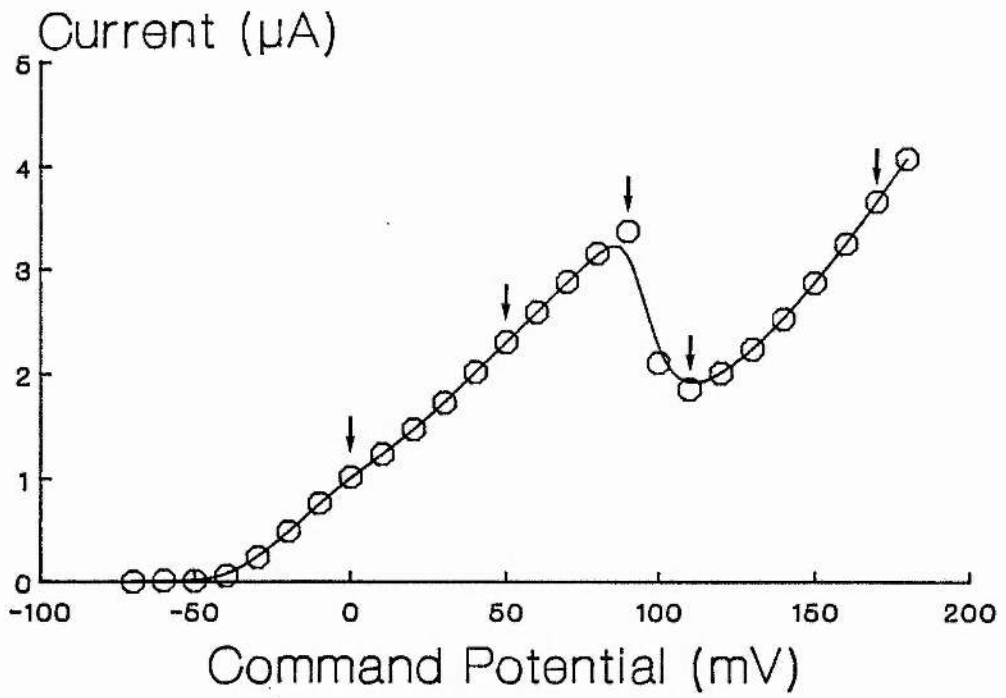


Fig. 3.211 2B

I-V relationship for the net outward currents.

The outward currents significantly developed at potentials greater than -50mV and increased linearly toward a maximum of 3.2μA around a command potential of +90mV. At potentials more positive than +90mV the currents rapidly declined toward a trough at +110mV before continuing to increase.

Arrows denote position of selected current responses: 0, +50, +80, +110 and +170mV.

3.212 Recording stability.

Most of the pharmacological experiments were completed within one hour. Impalements could, however, be maintained for much longer and an N-shape I-V relationship still observed. The experiment illustrated in Fig. 3.212 1A follows the current response over a three hour impalement. Selected current responses show that during this time the activation of the delayed rise time occurred at more negative potentials and the currents were increased at more positive potentials. This produced a -35mV shift in the activation of the hump together with a decline in the peak current by approximately 0.5uA and an increase in the currents associated with the upper arm of the curve (Fig. 3.212 1B). Furthermore, in this experiment the shift in the negative conductance region of the I-V relationship had reached a steady-state within 120mins. Currents in the upper arm region did, however, show a slight increase.

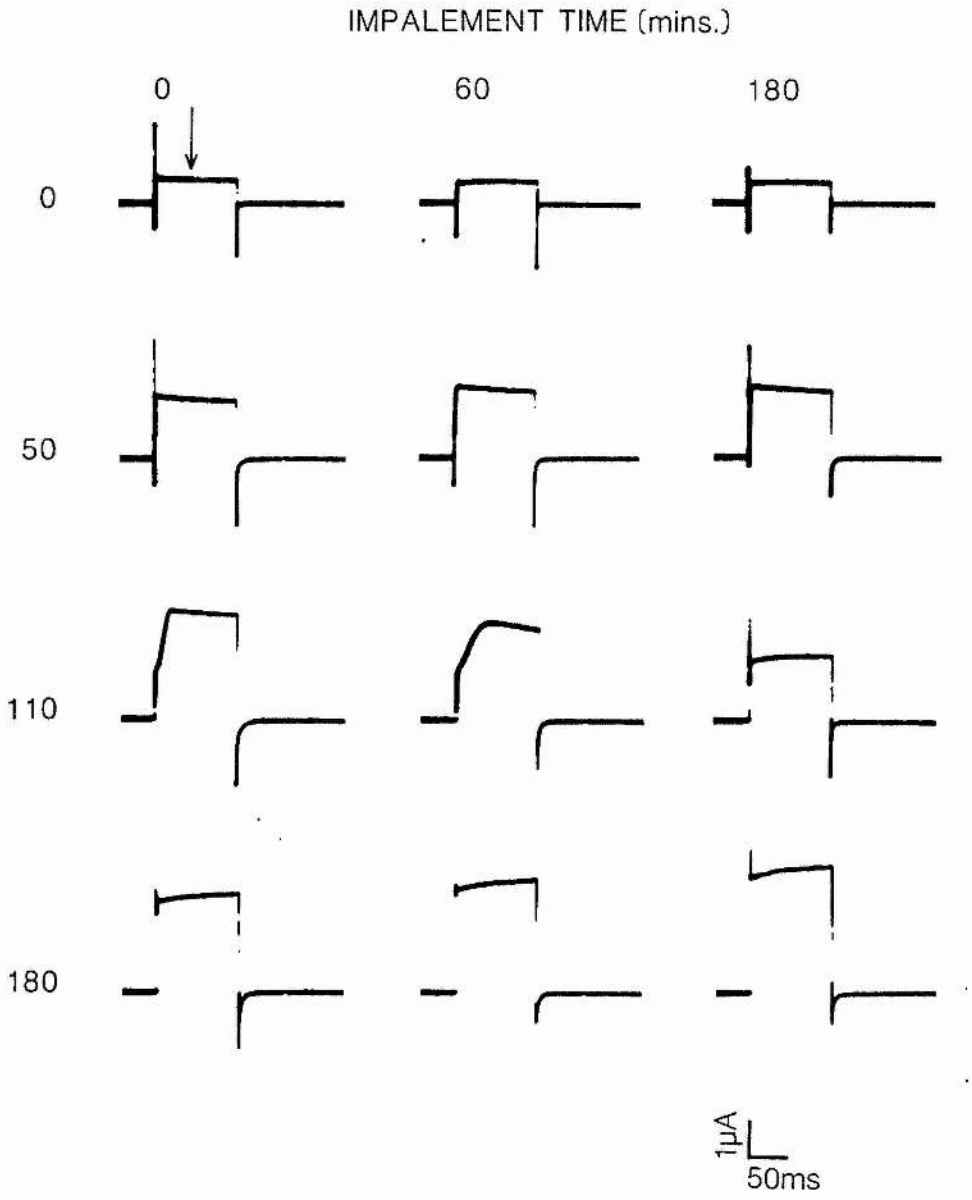


Fig. 3.212 1A

The effects of a long duration impalement on the net outward currents.

Selected current responses were taken at command potentials of 0, +50, +110, and +180mV after 0, 60, and 180mins. impalement.

Within 180mins. there was very little change in the overall magnitude and shape for currents at command potentials 0 and +50mV. For currents taken at command potentials of +110 there was an overall decline in magnitude during the first 120mins. of the experiment. While currents at a command potential of 180mV increased.

Arrow denotes the position of current measurements: 50ms.

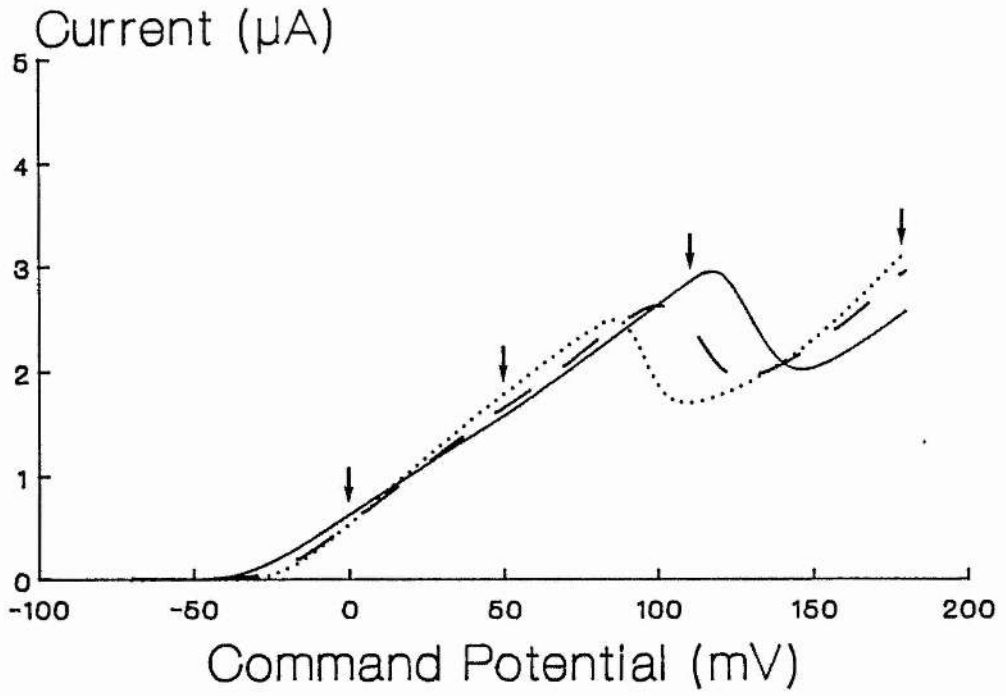


Fig. 3.212 1B

The effect a long duration impalement on the I-V relationship.

Curves represent time 0mins. (solid line), after 60mins. (dashed line) and after 180mins. impalement (dotted line).

Within 180mins. the hump gradually moved -35mV, and during this time there was also a 0.5μA reduction in the peak current.

Arrows denote the position of selected current responses: 0, +50, +110 and +180mV.

3.213 Leakage-current correction.

Non-specific ion movement through voltage-dependent channels in either direction during a command step may produce a leakage-conductance which is time and voltage-independent. The leakage-current is often small in comparison to the voltage-dependent currents and can be subtracted from the net current. In cell 3 this was achieved by applying positive and negative command steps of the same magnitude and duration and subsequently summating the current responses. This would remove a leakage component which was equal and opposite in either command potential polarity.

When this regime was applied to cell 3, hyperpolarising command potentials more negative than -200mV produced an inward current which rapidly increased with applied command steps and currents at more positive potentials were increased suggesting some form of membrane deterioration (Fig. 3.213 1A). The I-V curve of current measurements taken at 50ms clearly shows the large inward current at more negative potentials. The hump in the I-V relationship was still present (Fig. 3.213 1B).

The corrected current responses showed the delayed current rise and closely resembled the responses from the normal experimental run (Fig. 3.213 2A). The control run at the outset of the experiment is referred to as the normal experimental run. At potentials more positive than +130mV, however, the corrected outward current responses were reduced due to a large inward current flow during the corresponding hyperpolarising step. During subsequent voltage-clamp runs the outward currents were severely depressed with the N-shape reduced to a residual peak around +20 to +30mV (Fig. 3.213 2B). The outward currents remained suppressed until command potentials more positive than +110mV caused a sharp increase in current response. Command steps more positive than +150mV evoked currents in excess of

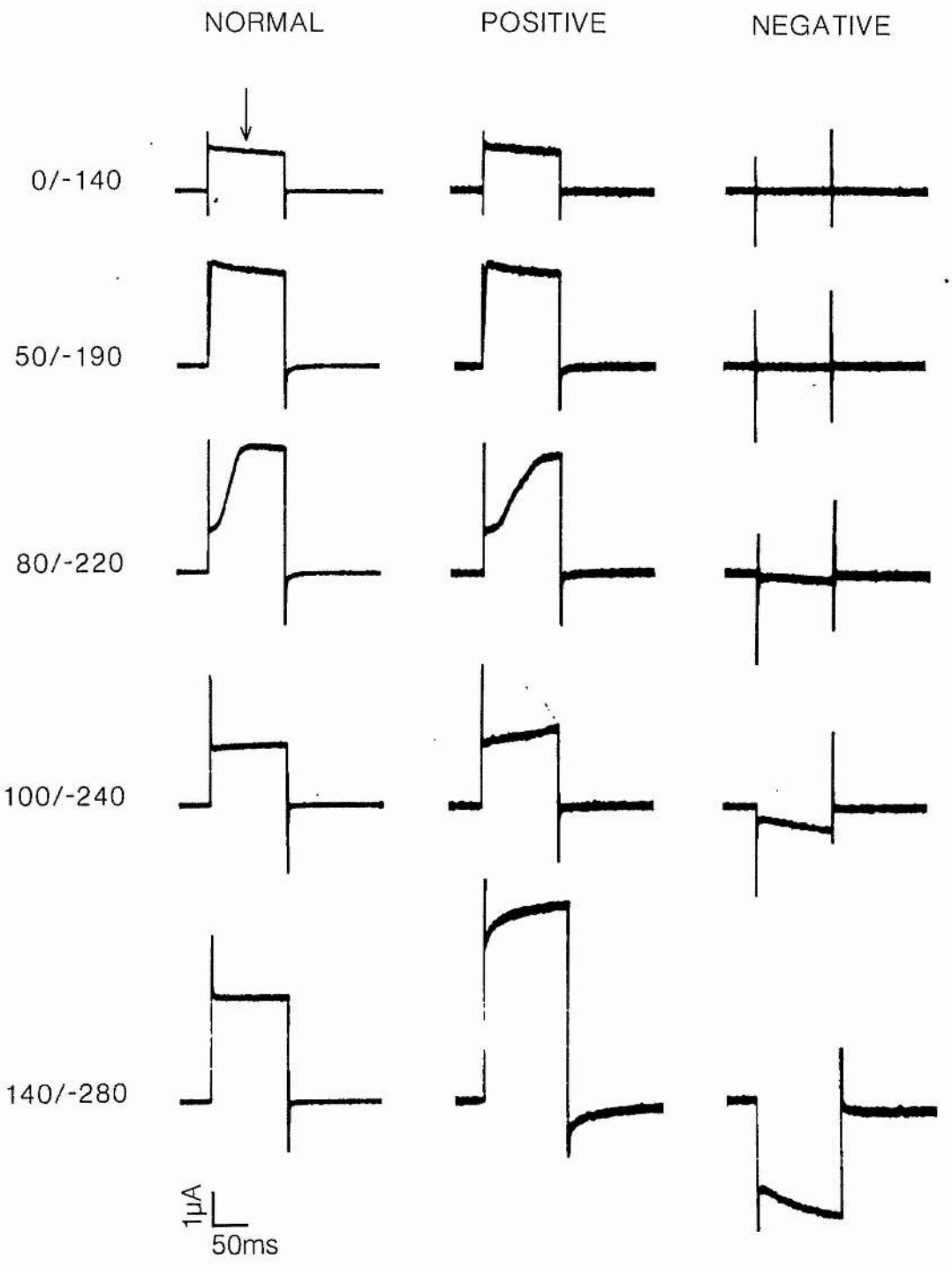
Fig. 3.213 1A

The effect of symmetrical positive and negative command pulses on the net current response.

Selected symmetrical current responses to positive and negative command potentials of 0/-140, +50/-190, +80/-220, +100/-240 and +140/-280mV.

The currents evoked from the positive command steps closely resemble currents from the normal run between potentials of -60 and +100mV. At command potentials more positive than +100mV, however, there was an increase in outward current. As command potentials became more negative than -190mV a significant inward current developed.

Holding potential: -70mV.


NORMAL
POSITIVE
NEGATIVE
0/-140
50/-190
80/-220
100/-240
140/-280
1μA
50ms

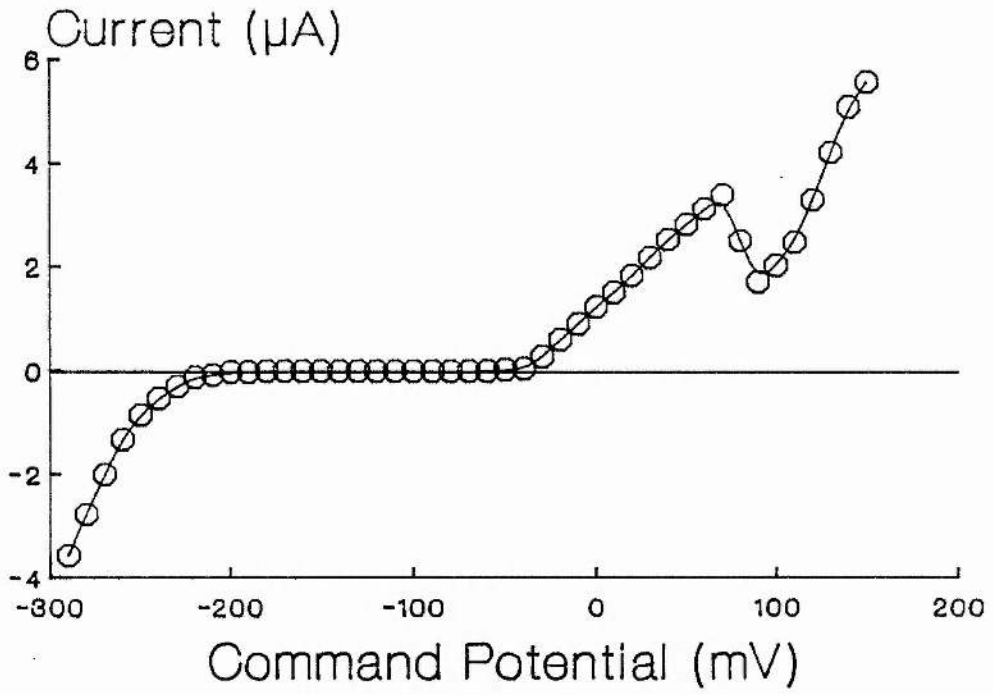


Fig. 3.213 1B

I-V relationship of the net current response to symmetrical positive and negative command pulses.

At positive potentials the curve resembled a normal run with the characteristic N-shape, although currents in the upper arm were larger. At potentials more negative than -190mV a large inward current was evoked.

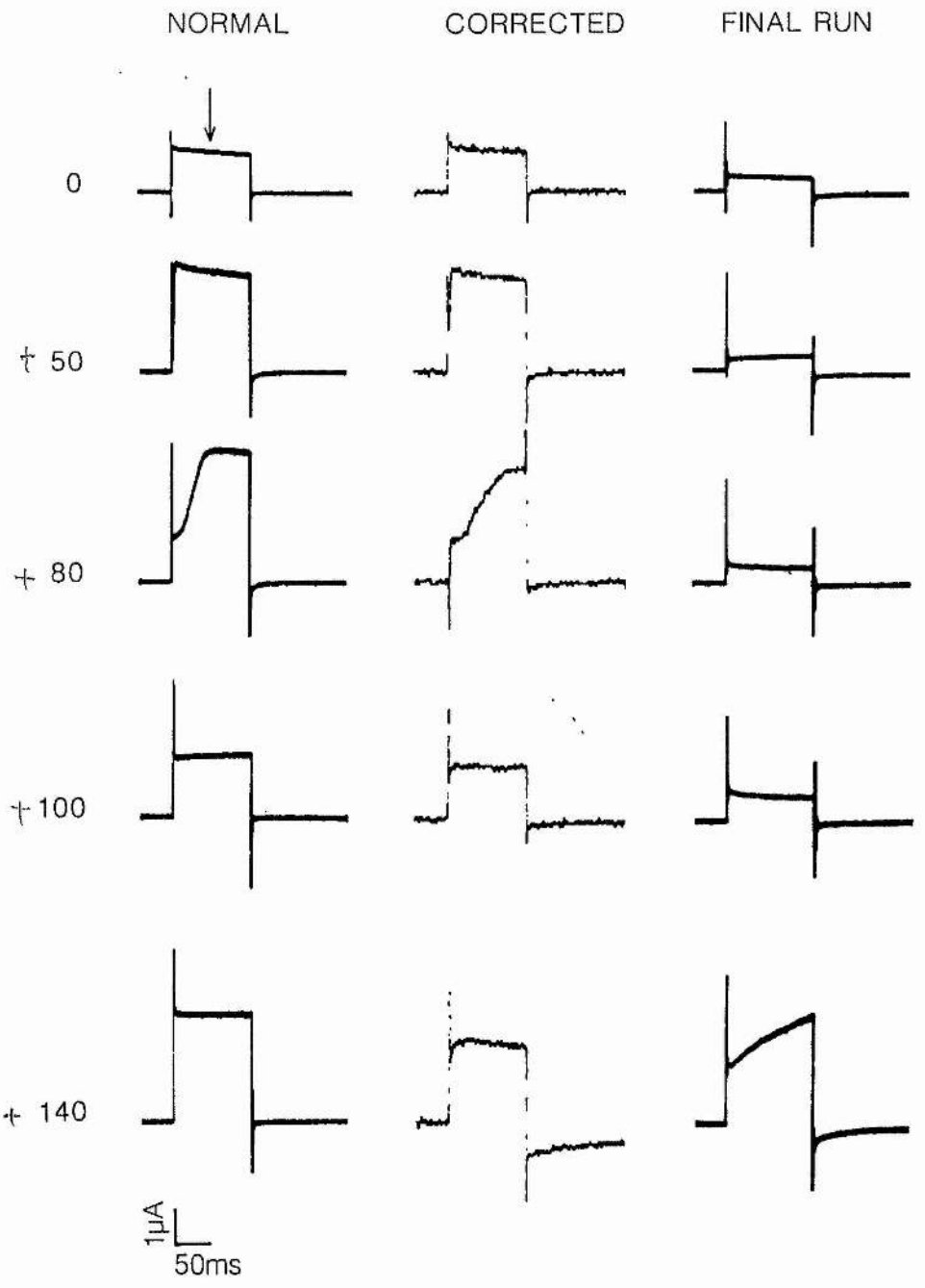
Fig. 3.213 2A

The effect of leakage current correction on the net outward current.

Selected current responses taken at command potentials of 0, +50, +80, +100 and +140mV.

The corrected run closely resembled the normal run between potentials of -60 and +100mV. Thereafter the response declined accompanied by large inward tail currents. Subsequent voltage-clamp runs were characterised by severely reduced currents. Command potentials more positive than +100mV evoked rapidly rising outward currents.

Holding potential: -70mV.


NORMAL
CORRECTED
FINAL RUN
0
+ 50
+ 80
+100
+ 140
1μA
50ms

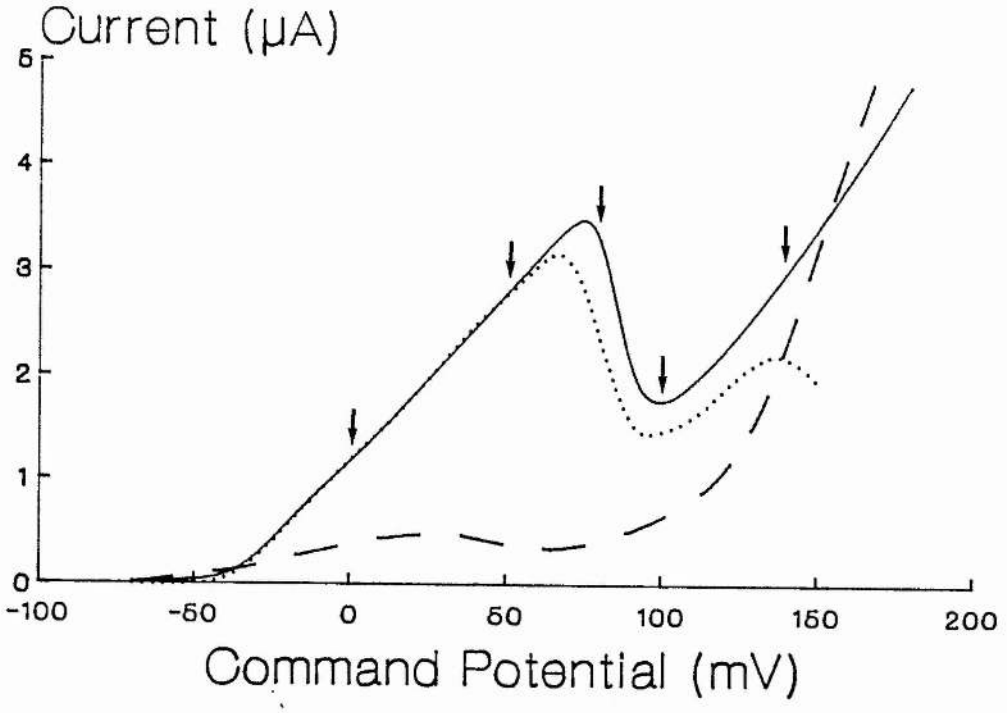


Fig. 3.213 2B

The effects of leakage current correction on the I-V relationship.

Curves represent a normal run (solid line), followed by the corrected response (dotted line) and finally, a normal run (dashed line).

The corrected curve closely followed the normal curve, though the negative conductance region was reduced in parallel to the normal run. At potentials more positive than +140mV the corrected current response declined. The final run produced severely reduced currents with a residual hump around +30mV and currents rising sharply at potentials more positive than +110mV.

Arrows denote the position of selected current responses: 0, +50, +80, +100 and +140mV.

the original normal response.

In view of the close adherence of the corrected curve to the uncorrected curve at potentials more negative than +130mV, and the disruption of subsequent current responses, leakage current correction was not routinely performed. In addition, Fig. 3.213 1B shows that the leakage current between -180 and -70mV was small compared with the large voltage-dependent conductance changes which occur at potentials more positive than -50mV.

3.22 PHARMACOLOGICAL CHARACTERISATION OF THE NET OUTWARD CURRENTS.

The outward currents were then characterised in terms of the three classical outward currents (I_A I_{KCa} and I_K) found in a variety of preparations (see section 1.22) and in particular molluscan neurones (Thompson, 1977; Adams, Smith and Tompson, 1980). In addition, a preliminary search was made for any chloride ion contribution.

3.221 Early, fast transient current.

In molluscs the early fast transient current, I_A, can be separated from the late outward curents I_{KCa} and I_K because of its distinct kinetics and pharmacology. I_A is activated at more negative command potentials than I_{KCa} or I_K (Connor and Stevens, 1971b) and is sensitive to aminopyridine blockade (Thompson, 1977).

3.2211 Altering the holding potential.

Fig. 3.2211 1A shows the effect of altering the holding potential on the activation of outward currents. There was very little difference in the shape of the current response between V_H -90 and -70mV and the I-V relationship for both holding potentials superimpose (Fig. 3.2211 1B). If an early transient inactivating outward current similar to I_A were significantly present in this cell, holding at -90mV and stepping to potentials less negative than -30mV would have been likely to produce a fast transient current (Connor and Stevens, 1971b). However, under those conditions there was no evidence of a fast transient component in cell 3 (Fig. 3.2211 1A). As the holding potential became more positive, i.e. approached -30mV, there was an overall reduction in the outward current and I-V relationship with a concomitant shift in the activation of the hump toward more negative potentials (Fig. 3.2211 1B). In addition, holding at -30mV activated a significant steady-state current which accounted for the parallel shift in the lower arm of the I-V curve. In effect the base line had

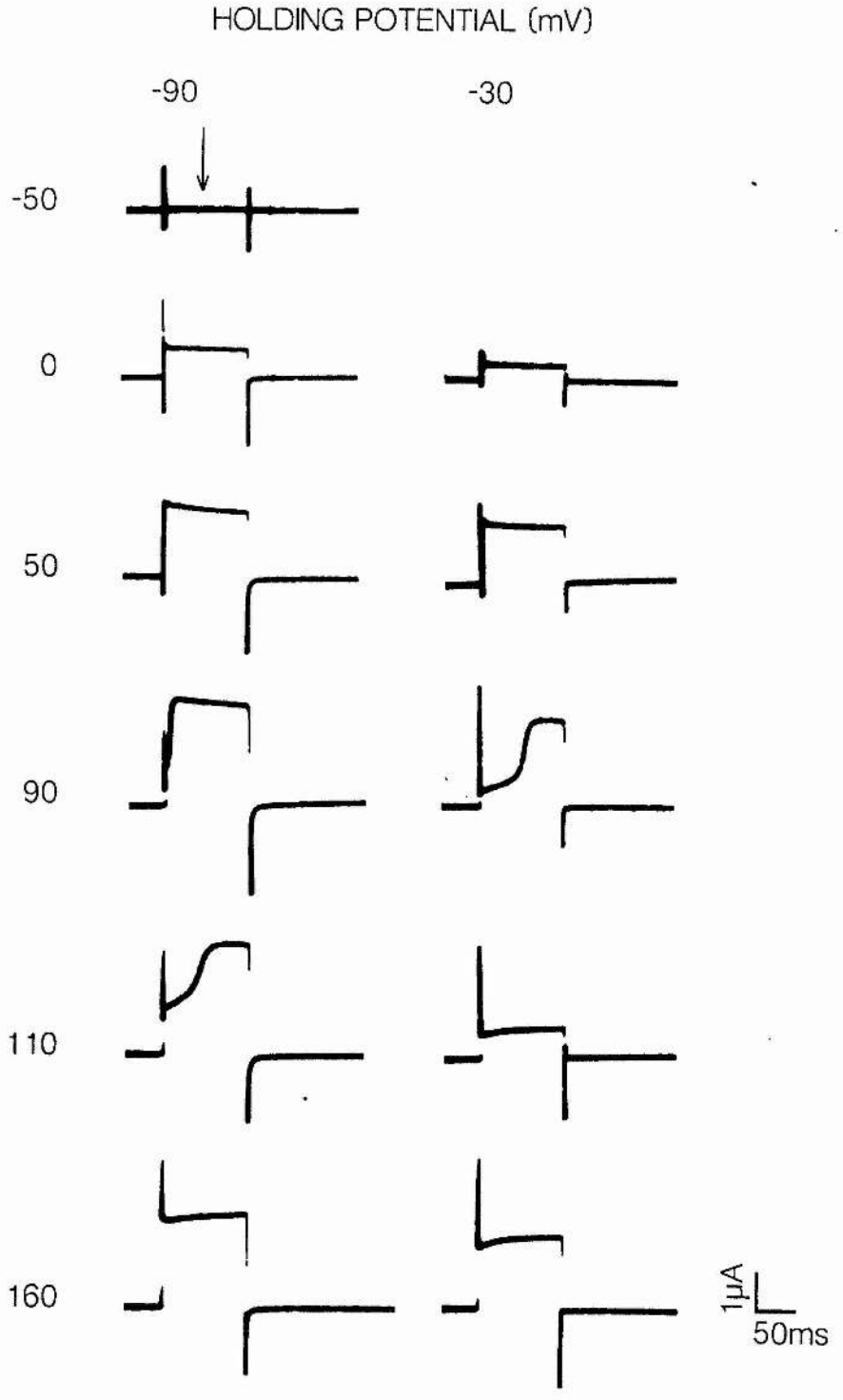
Fig. 3.2211 1A

The effect of altering the holding potential on the net outward current.

Selected current responses taken at command potentials of -50, 0, +50, +90, +110 and +160mV.

There was no evidence for an early, fast transient current component activated by depolarisation from a holding potential of -90mV to command potentials less positive than -50mV. Holding at more positive potentials shifted the delayed current rise toward more negative potentials.

Arrow denotes the position of current measurements: 50ms.


HOLDING POTENTIAL (mV)
-90
-30
-50
0
50
90
110
160
1μA
50ms

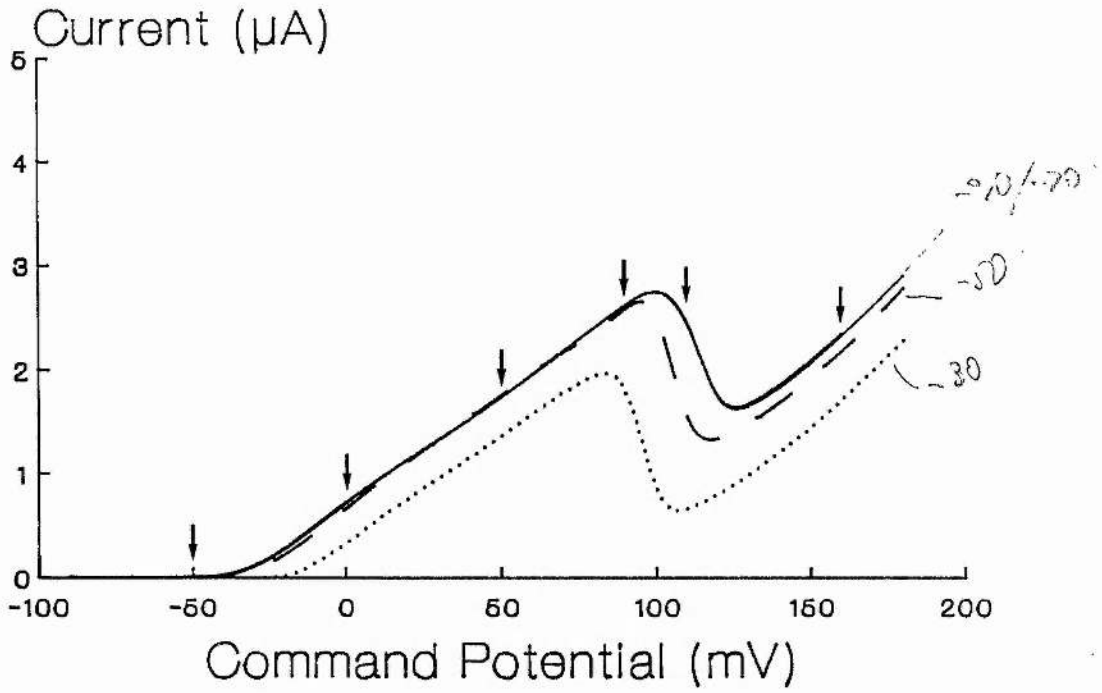


Fig. 3.2211 1B

The effect of altering the holding potential on the I-V relationship.

Curves represent holding potentials of -90 and -70mV (superimposed solid line), -50mV (dashed line) and -30mV (dotted line).

There was no significant difference in current values for holding potentials of -90 or -70mV. As the holding potential became more positive the hump moved toward more negative potentials and the peak current was reduced. Holding at -30mV activated a significant steady-state current which resulted in a parallel reduction of the I-V curve.

Arrows denote the position of selected current responses: -50, 0, +50, +90, +110 and +160mV.

been raised.

3.2212 Aminopyridines.

External application of neither 4-AP (5mM) nor 3,4-DAP (5mM) had a detectable effect on the early portion of the current response but caused the delayed current rise to occur at more negative potentials (Figs. 3.2212 1A and 3.2212 2A respectively). The corresponding I-V relationships showed a reduction in the activation of the hump and a concomitant shift toward more negative potentials (Fig 3.2212 1B and 3.2212 2B). At similar concentrations 3,4-DAP was marginally more effective that 4,AP in shifting the I-V relationship. The aminopyridines had only a minor effect on the currents in the lower arm of the I-V curve In some but not all preparations, however, aminopyridines caused a small increase in currents associated with the lower arm of the curve (not shown). The maximum current increase was 0.25μA (with 5mM 4-AP) which was restored to normal after washout with normal saline.

Fig. 3.2212 1A

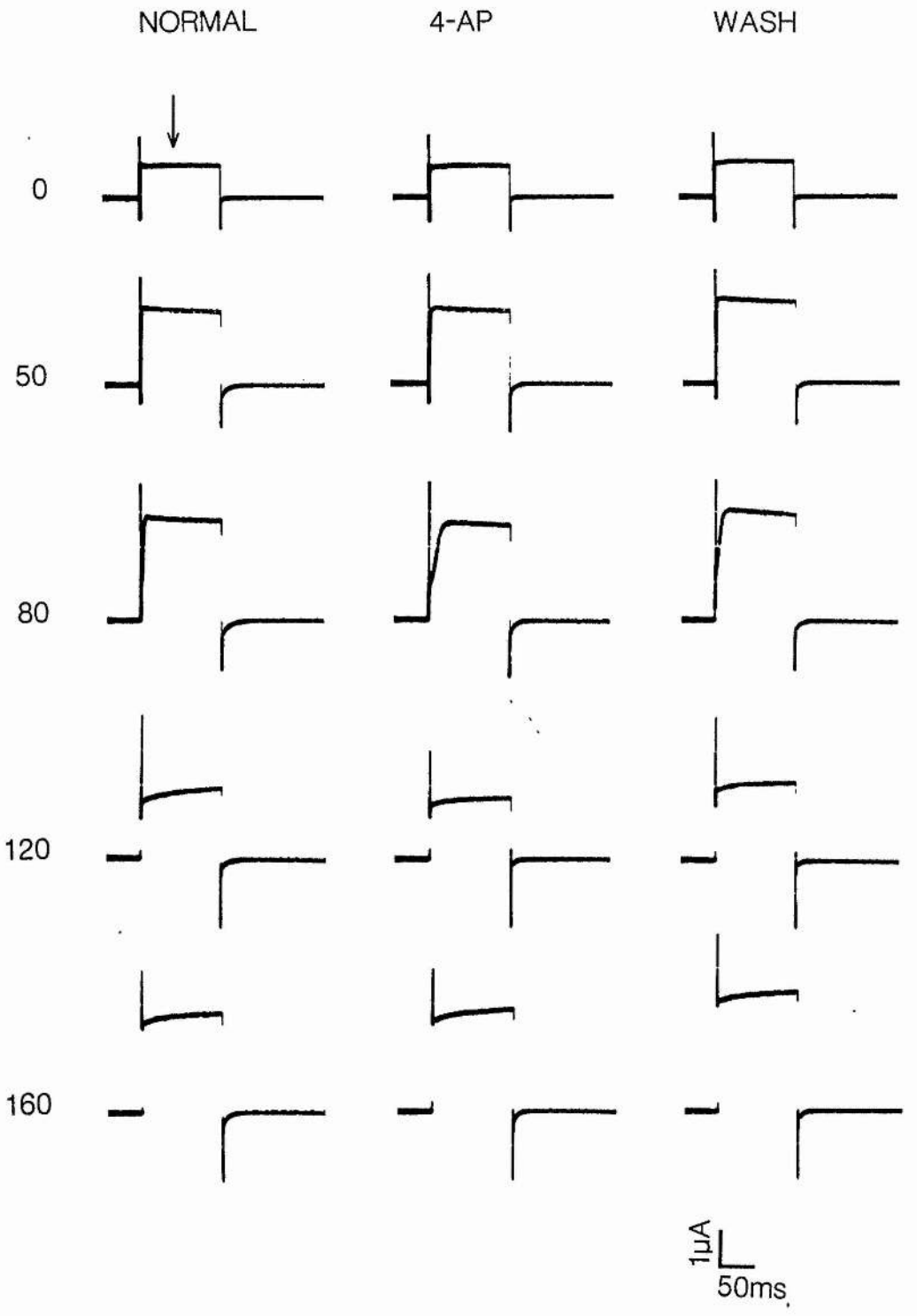
The effect of 4-AP on the net outward current.

Selected current responses taken at command potentials of 0, +50, +80, +120, and +160mV.

After 20mins. 4-AP (5mM) had no significant effect on the current response between command potentials of -60 and +50mV. The delayed current rise did, however, occur at more negative command potentials.

Holding potential: -70mV.

Arrow denotes the position of current measurements: 50ms.


NORMAL
4-AP
WASH
0
50
80
120
160
1μA
50ms

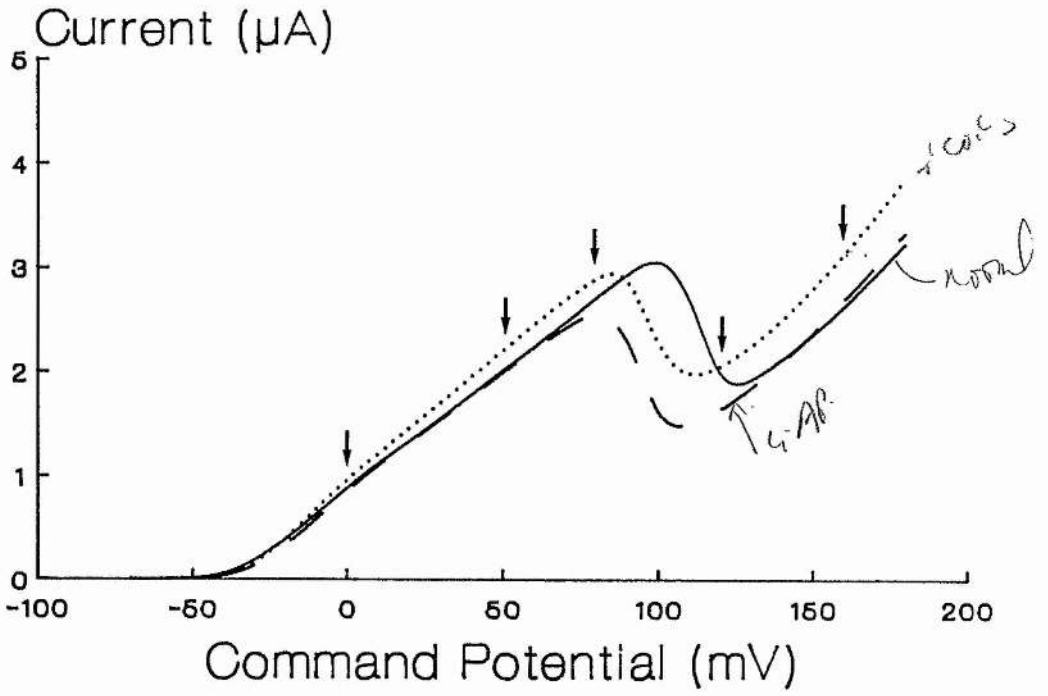


Fig. 3.2212 1B

The effect of 4-AP on the I-V relationship.

Curves represent normal run (solid line) followed by 10mins. 4-AP (5mM) (dashed line) and finally, 20mins. wash with normal saline (dotted line).

In this experiment 4-AP shifted the hump -25mV and reduced the peak current by 0.6μA. These effects were partially reversed after washing with normal saline.

Arrows denote the position of selected current traces: 0, +50, +80, +120 and +160mV.

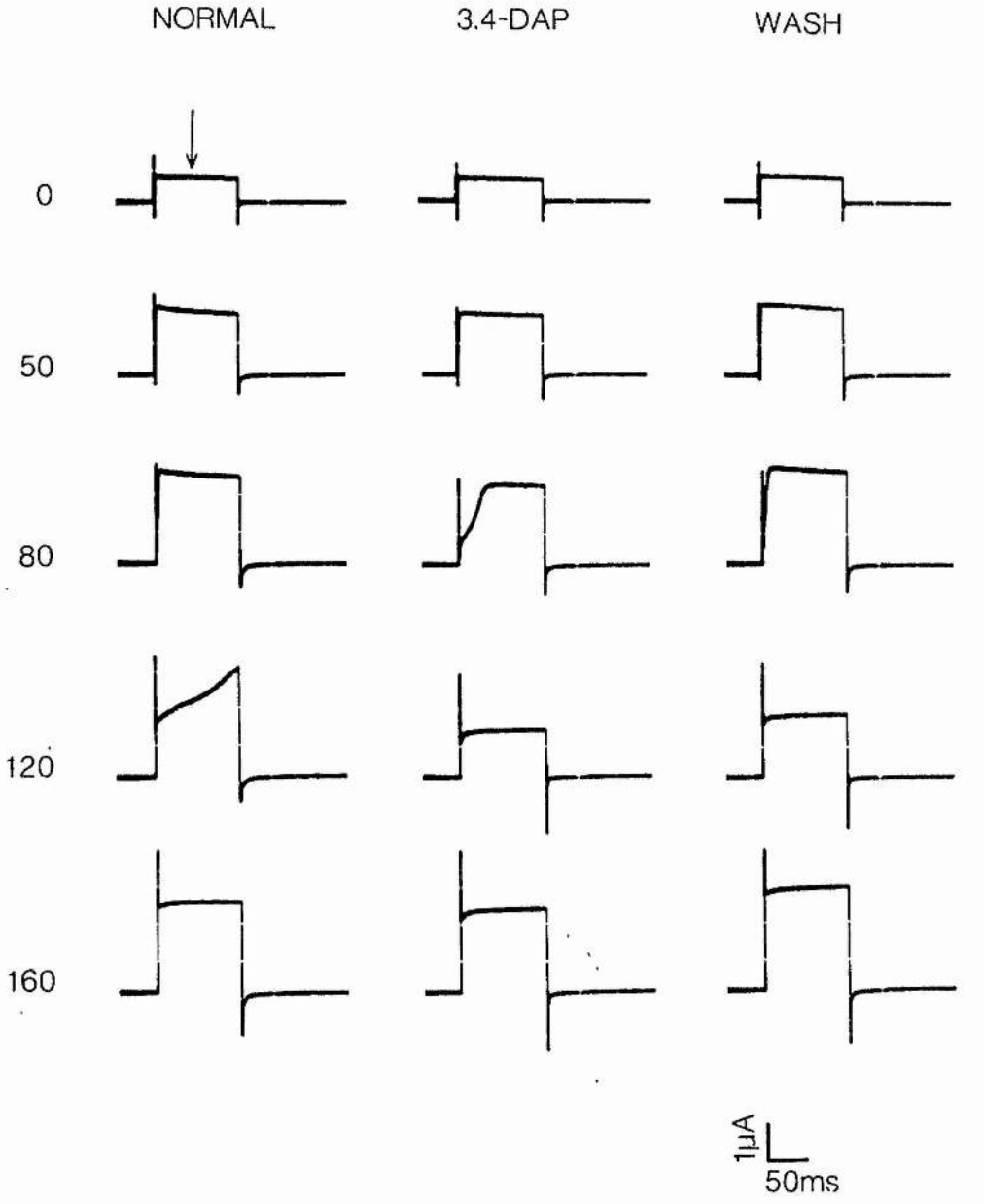


Fig. 3.2212 2A

The effect of 3,4-DAP on the net outward currents.

Selected current responses taken at command potentials of 0, +50, +80, +120 and +160mV.

Within 10mins. 3,4-DAP caused a slight reduction in currents between command potentials of -60 and +80mV and the delayed current rise now occurred at more negative command potentials. These effects were partially reversed after 10mins. wash with normal saline.

Holding potential:-70mV.

Arrow denotes the position of current measurements: 50ms.

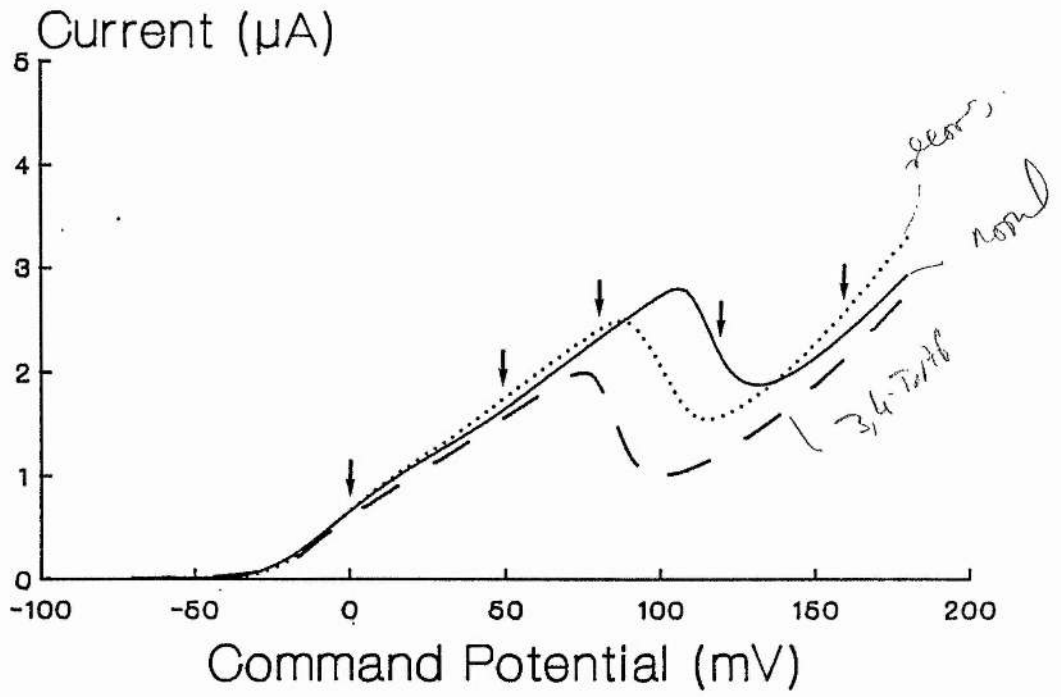


Fig. 3.2212 2B

The effect of 3,4-DAP on the I-V relationship.

Curves represent normal run (solid line) followed by 10mins. 3,4-DAP (5mM) (dashed line) and finally, 10mins. wash with normal saline (dotted line).

In this experiment 3,4-DAP shifted the hump -30mV and reduced the peak current by 0.9μA. Currents in the upper arm were also reduced. These effects were partially reversed after washing with normal saline.

Arrows denote the position of selected current responses: 0, 50, 80, 120 and 160mV.

3.222 The involvement of calcium in the outward currents.

The N-shape I-V relationship is characteristic of a calcium-dependent outward current originally observed in molluscan neurones (Meech and Standen, 1974; 1975) and subsequently identified in a number of different preparations (see section 1.223) including an identified insect motoneurone (Thomas, 1984). The involvement of calcium in the net-outward currents of cell 3 initially involved altering the external calcium ion concentration and later using inorganic and organic calcium blockers and a toxin blocker specific for a sub-type of I_{KCa}.

3.2221 Altering the external calcium ion concentration.

Increasing the external calcium ion concentration between 4.5mM and 36mM produced an increase in the net outward current response underlying the delayed current rise (FIg. 3.2221 1A). This was observed on the corresponding I-V curve as an increase in the peak hump currents and negative conductance region with respect to the curve at normal calcium concentration (9mM) (Fig. 3.2221 1B). In this experiment there was a concomitant +20mV shift in the activation of the hump from +80mV to +100mV.

3.2222 Inorganic calcium blockers: Cd^{2+} and Mn^{2+}.

Certain polyvalent cations e.g. Cd^{2+} and Mn^{2+} competitively block calcium binding sites. In these experiments external Cd^{2+} (1mM) and Mn^{2+} (5 mM) reversibly abolished the current underlying the delayed rise (Figs. 3.2222 1A and 3.2222 2A respectively) and consequently the hump in the I-V curve (Figs. 3.2222 1B and 3.2222 2B).

An activation curve for the Cd^{2+}- and Mn^{2+}-sensitive current component was derived by subtracting the current values in the presence of the inorganic blockers from the normal values before blockade. The curve had a bell shape (Figs. 3.2222 1C and 3.2222 2C) with a peak between +100 and +130mV. In this experiment cadmium also

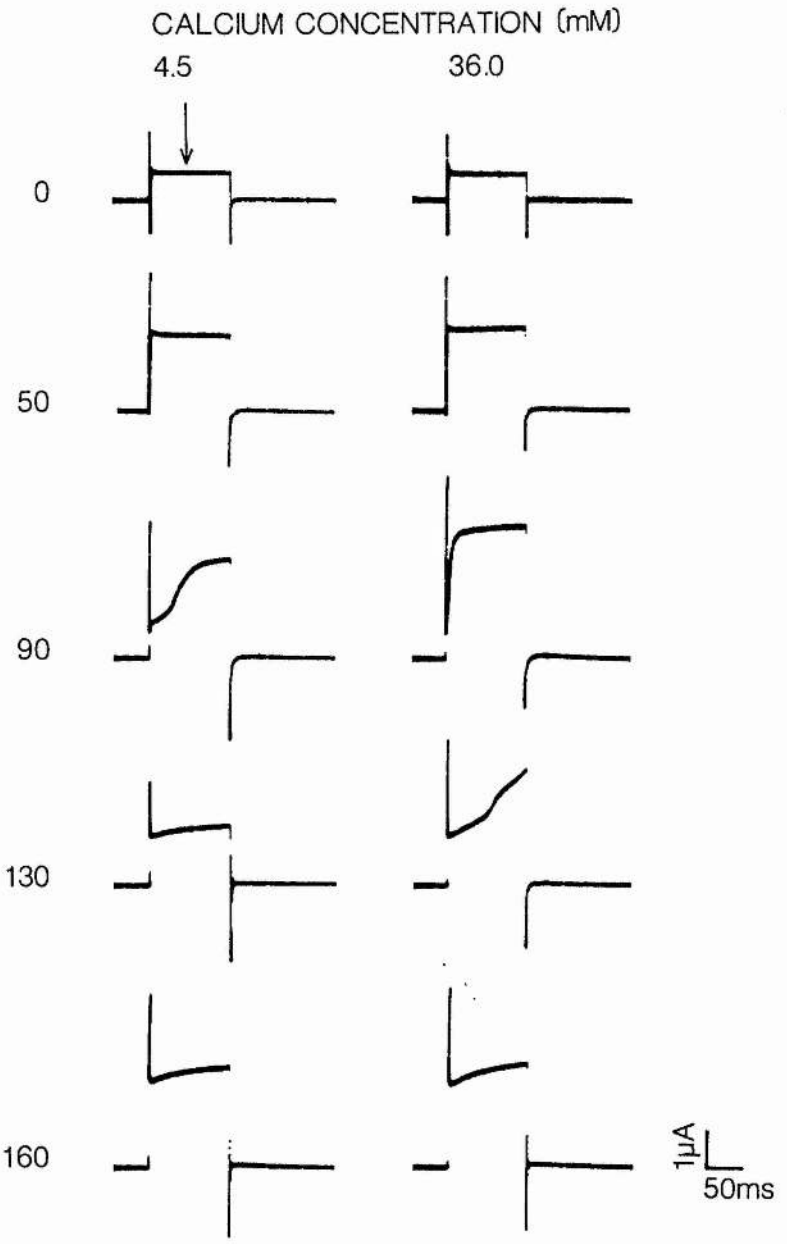


Fig. 3.2221 1A

The effect of altering the external calcium ion concentration on the net outward currents.

Selected current responses taken at command potentials of 0, +50, +90, +130 and +160mV.

Increasing the external calcium ion concentration from 4.5mM to 36.0mM caused an increase in outward currents around command potentials of +90mV and shifted the delayed current rise to more positive potentials.

Holding potential: -70mV.

Arrow denotes the position of current measurements: 50ms.

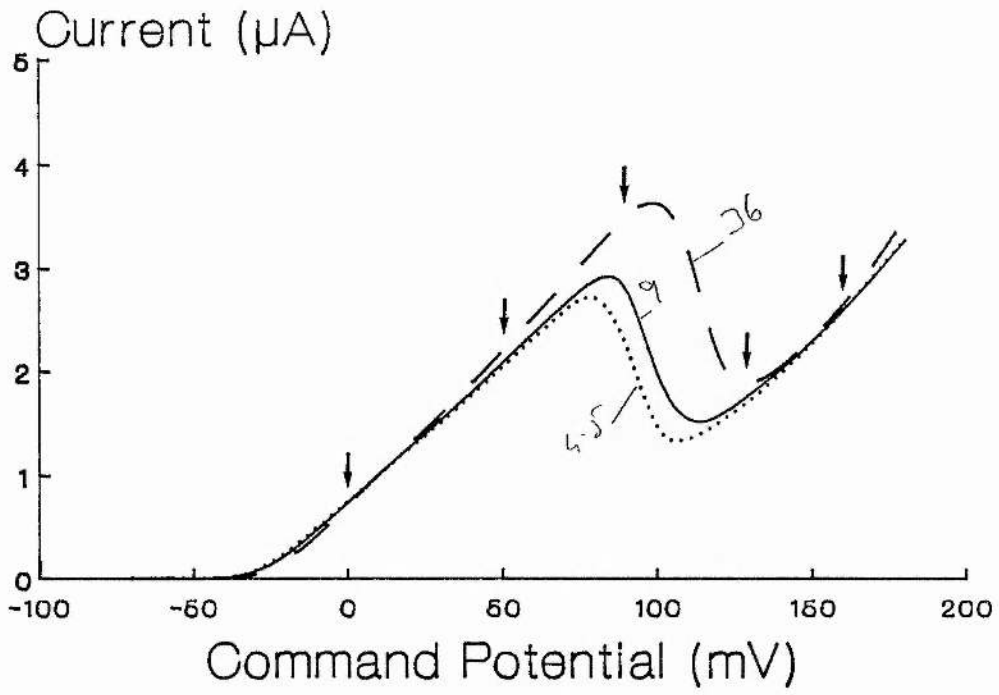


Fig. 3.2221 1B

The effect of altering the external calcium ion concentration on the I-V relationship.

Curves represent external calcium concentrations of 4.5 (dotted line), 9.0 (solid line) and 36.0mM (dashed line).

Increasing the calcium ion concentration caused a +20mV shift in the hump and an increase in associated currents. The magnitude of the negative conductance region was also increased.

Arrows denote position of selected current responses: 0, +50, +90, +130 and +160mV.

Fig. 3.2222 1A

The effect of cadmium on the net outward current.

Selected current responses taken at command potentials of 0, +50, +100, +130 and +160mV.

Within 10mins. cadmium (1mM) completely abolished the current underlying the delayed rise and caused an overall reduction in outward currents. These effects were partially reversed after 25mins. wash with normal saline.

Holding potential: -70mV.

Arrow denotes position of current measurements: 50ms.

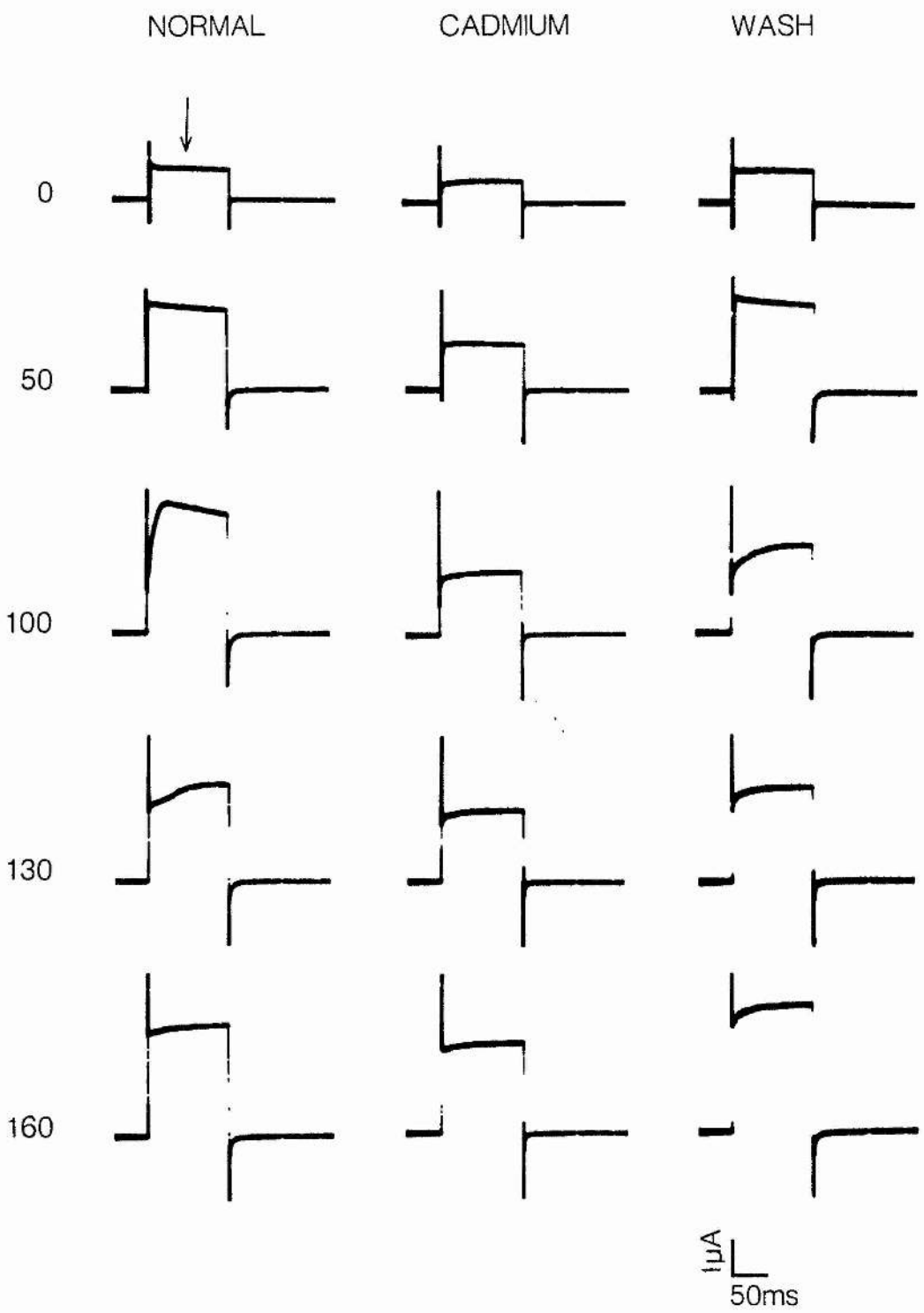

NORMAL
CADMIUM
WASH
0
50
100
130
160
1μA
50ms

Fig. 3.2222 1B

The effect of cadmium on the I-V relationship.

Curves represent normal run (solid line) followed by 10mins cadmium (1mM) (dashed line) and finally, after 25mins. wash with normal saline (dotted line).

Cadmium completely abolished the hump and reduced currents in the upper arm region. These effects were partially reversed after washing with normal saline.

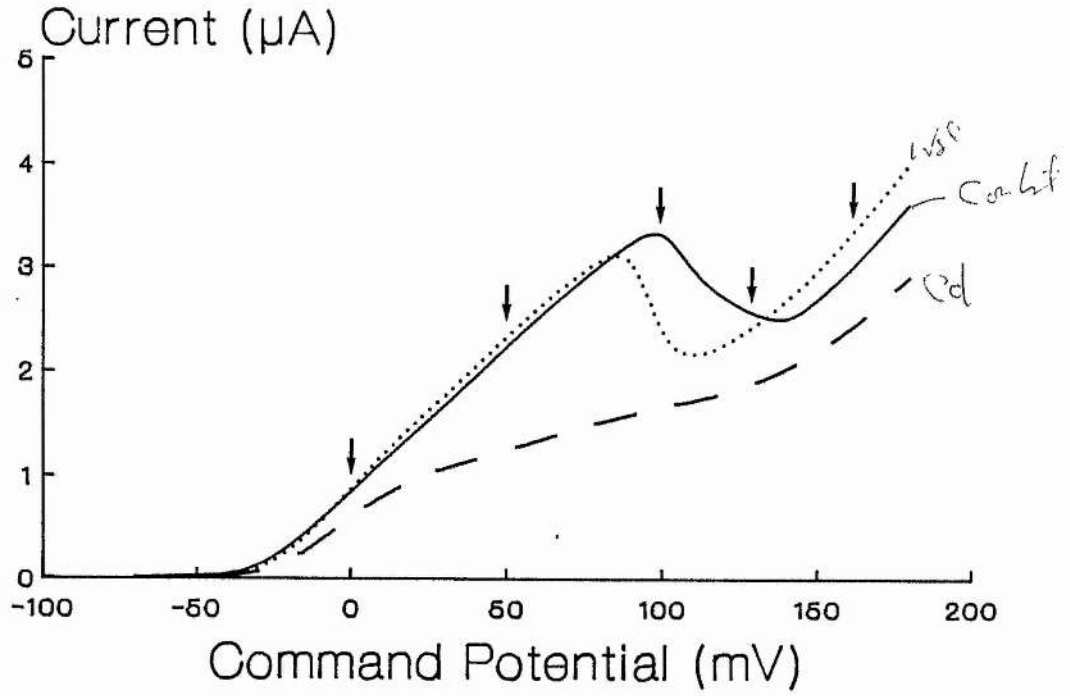
Arrows denote position of selected current responses: 0, +50, +100, +130 and +160mV.

Fig. 3.2222 1C

The activation curve for the cadmium-sensitive current component.

The bell-shaped activation curve peaked around +100mV and had a projected voltage axis intersect of between +150mV and +160mV.

B


Current (μA)
5
4
3
2
1
0
-100
-50
0
50
100
150
200
Command Potential (mV)
Cd

C

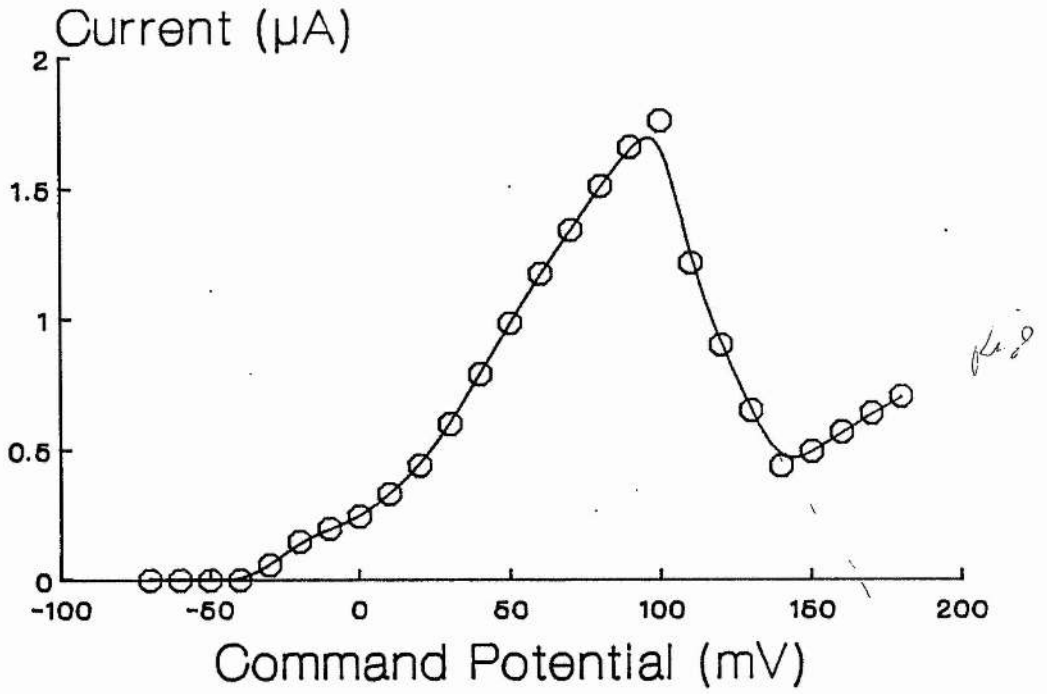

Current (μA)
2
1.5
1
0.5
0
-100
-50
0
50
100
150
200
Command Potential (mV)

Fig. 3.2222 2A

The effect of manganese on the net outward currents.

Selected current responses taken at command pontentials of 0, +50, +100, +120 and +160mV.

Within 10mins. manganese (5mM) reduced currents between command potentials of -60 and +80mV and abolished the current underlying the delayed rise. These effects were reversed after 10mins. wash with normal saline.

Holding potential: -70mV

Arrow denotes position of current measurements: 50ms.

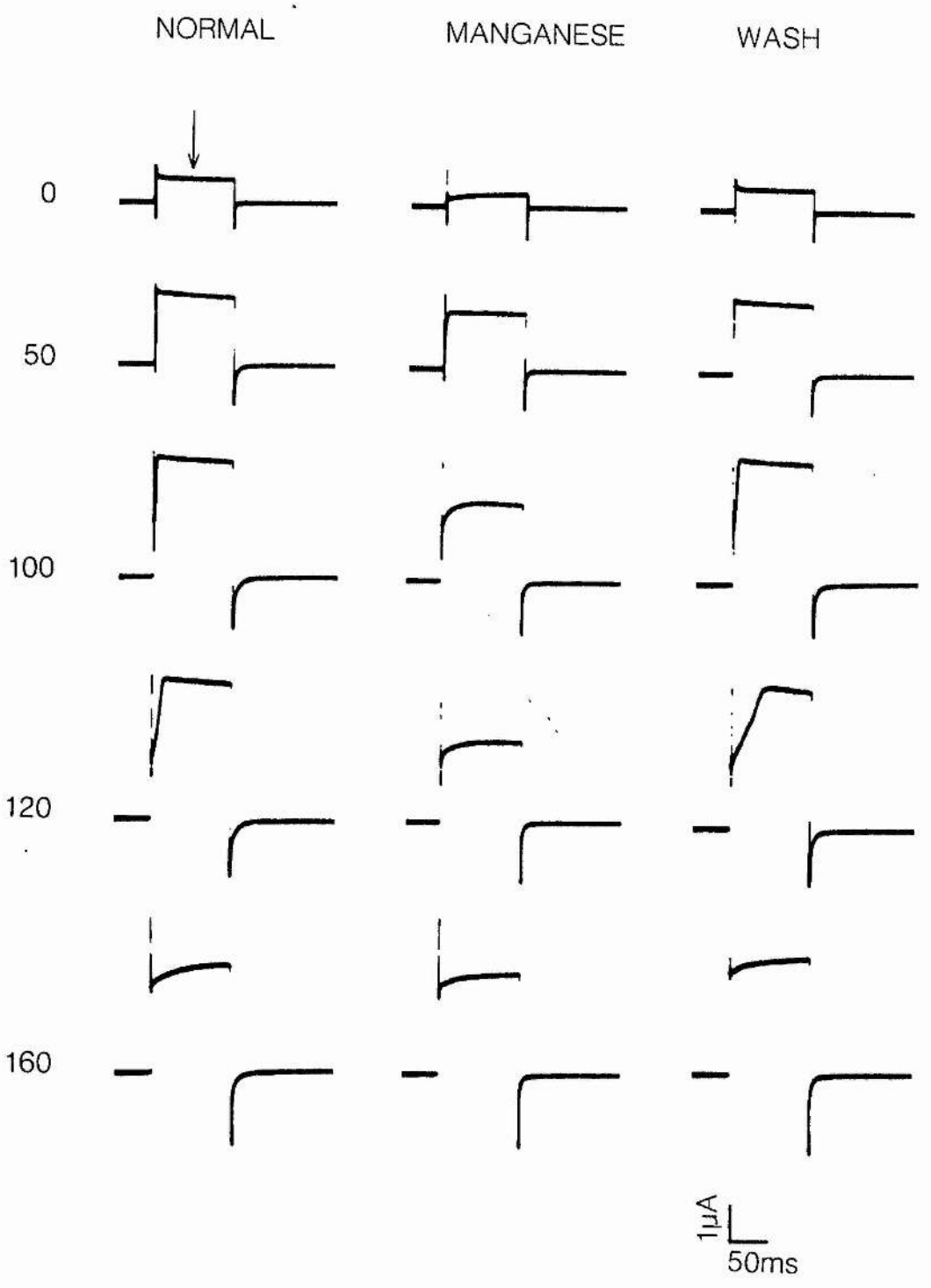

NORMAL
MANGANESE
WASH
0
50
100
120
160
1μA
50ms

Fig. 3.2222 2B

The effect of manganese on the I-V relationship.

Curves represent normal run (solid line) followed by 10mins. manganese (5mM) (dashed line) and finally, after 10mins. wash with normal saline (dotted line).

Within 10mins. manganese reduced the hump to a residual peak around +80mV. These effects were reversed after washing with normal saline.

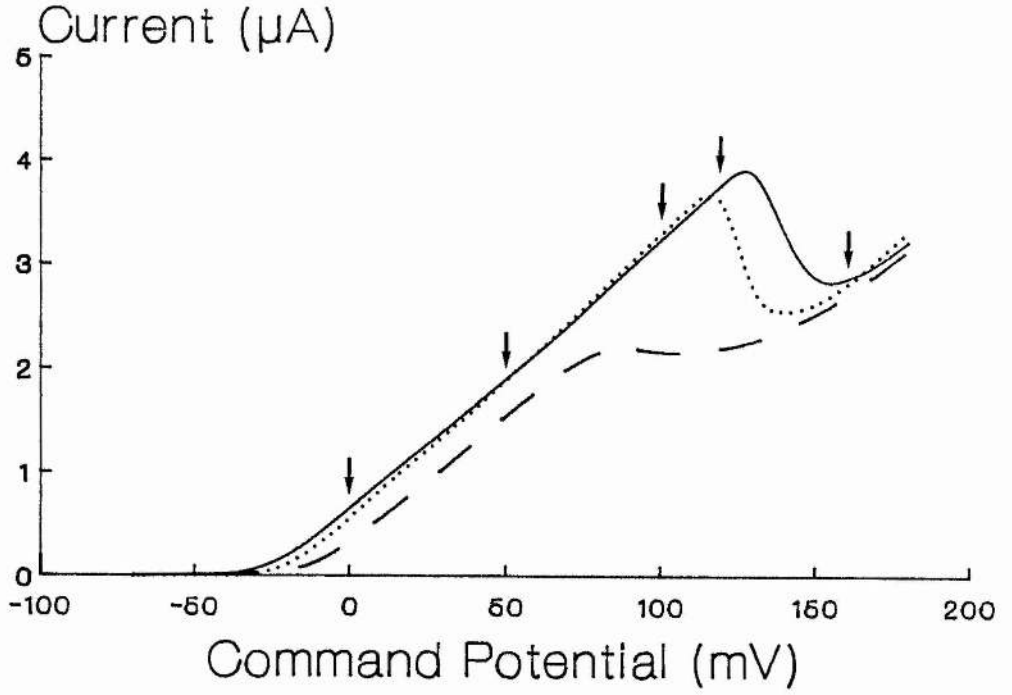
Arrows denote position of selected current responses: 0, +50, +100, +120 and +160mV.

Fig. 3.2222 2C

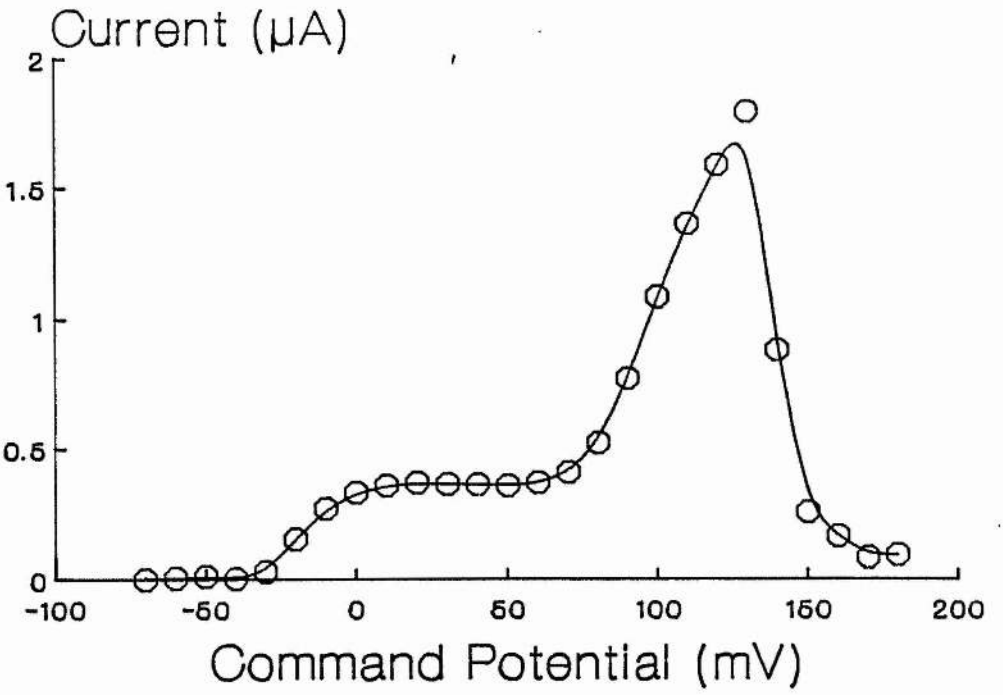
The activation curve for the manganese sensitive current component.

The bell-shaped activation curve peaked around +125mV and had a projected voltage axis intersect of between +150mV and +160mV.

B


Current (μA)
6
4
3
2
1
0
-100
-50
0
50
100
150
200
Command Potential (mV)

C


Current (μA)
2
1.5
1
0.5
0
-100
-50
0
50
100
150
200
Command Potential (mV)

suppressed a current component at more positive potentials. This was not a consistent feature of Cd^{2+} blockade where, in some experiments, the currents in the upper arm region were unaffected (compare with Fig. 3.2232 1B). Consequently the cadmium-sensitive activation curve began to rise at command potentials more positive than 140mV whereas the manganese-sensitive activation curve gradually declined. The manganese-sensitive activation curve also has a shoulder between command potentials of 0 and +100mV whereas the cadmium-sensitive curve does not. This could be attributed to the stage of blockade achieved by manganese within 10mins. i.e. incomplete suppression of the hump, rather than a different underlying blockade mechanism. In this preparation Cd^{2+} appeared to be more effective in abolishing the hump than comparable concentrations of Mn^{2+}.

The projected intersect of the voltage axis made by the activation curves should represent the calcium equilibrium potential. As the command potential approaches the calcium equilibrium potential the driving force on external Ca^{2+} should decrease and no more Ca^{2+} is able to enter the cell to activate the outward current. For the cadmium-sensitive component the voltage intersect was difficult to discern because of the late rise in currents beyond the command potential of +140mV. However, the projected intersect lay between +150 and +160mV. The intersect of the manganese-sensitive component also lay between +150 and +160mV . This value agrees closely with the intersect at +140mV of a similar calcium-dependent outward current reported in molluscan neurones (Meech, 1978). Some variation may exist between preparations and this relates to the time dependency of current measurements. For the extrapolation to be a more accurate measurement of the calcium equilibrium potential, current measurements ought to have been taken at the maximal current

response during a longer duration command pulse before and after blockade.

3.2223 Organic calcium blocker: verapamil.

At low concentrations (50-200μM) the organic calcium antagonist verapamil hydrochloride (5-[(3,4-Dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride) abolished the delayed current rise and caused an overall reduction in the net outward currents (Fig. 3.2223 1A). For command potentials between +20 and +110mV the block appeared to be time-dependent. Currents gradually fell during the command pulse indicating an increased rate of inactivation for the remaining current (arrowhead). Fig. 3.2223 2A shows that prolonged exposure to verapamil (100uM) caused a further increase in inactivation of the remaining current which was more apparent for long duration command pulses. The hump of the I-V curve was reduced but not abolished (Fig. 3.2223 1B) so that a residual hump remained around +70mV. Verapamil reduced the magnitude of the negative conductance region thereby flattening the curve. In addition, prolonged exposure to verapamil caused a greater suppression of the hump in the I-V relationship (Fig. 3.2223 2B), leaving a small residual hump around command potentials of +30 to +40mV.

With prolonged exposure verapamil appeared to partially block not only the calcium-dependent current but also an additional current component. Total block of the hump was achieved at high concentrations (200 - 500μM) but the block was then difficult to reverse and washout was often accompanied by increased currents in the upper arm region of the I-V curve (not shown), suggesting damage to the neuronal membrane.

3.2224 Toxin I_{KCa} blocker: apamin.

Application of the bee venom toxin, apamin, at concentrations up to

Fig. 3.2223 1A

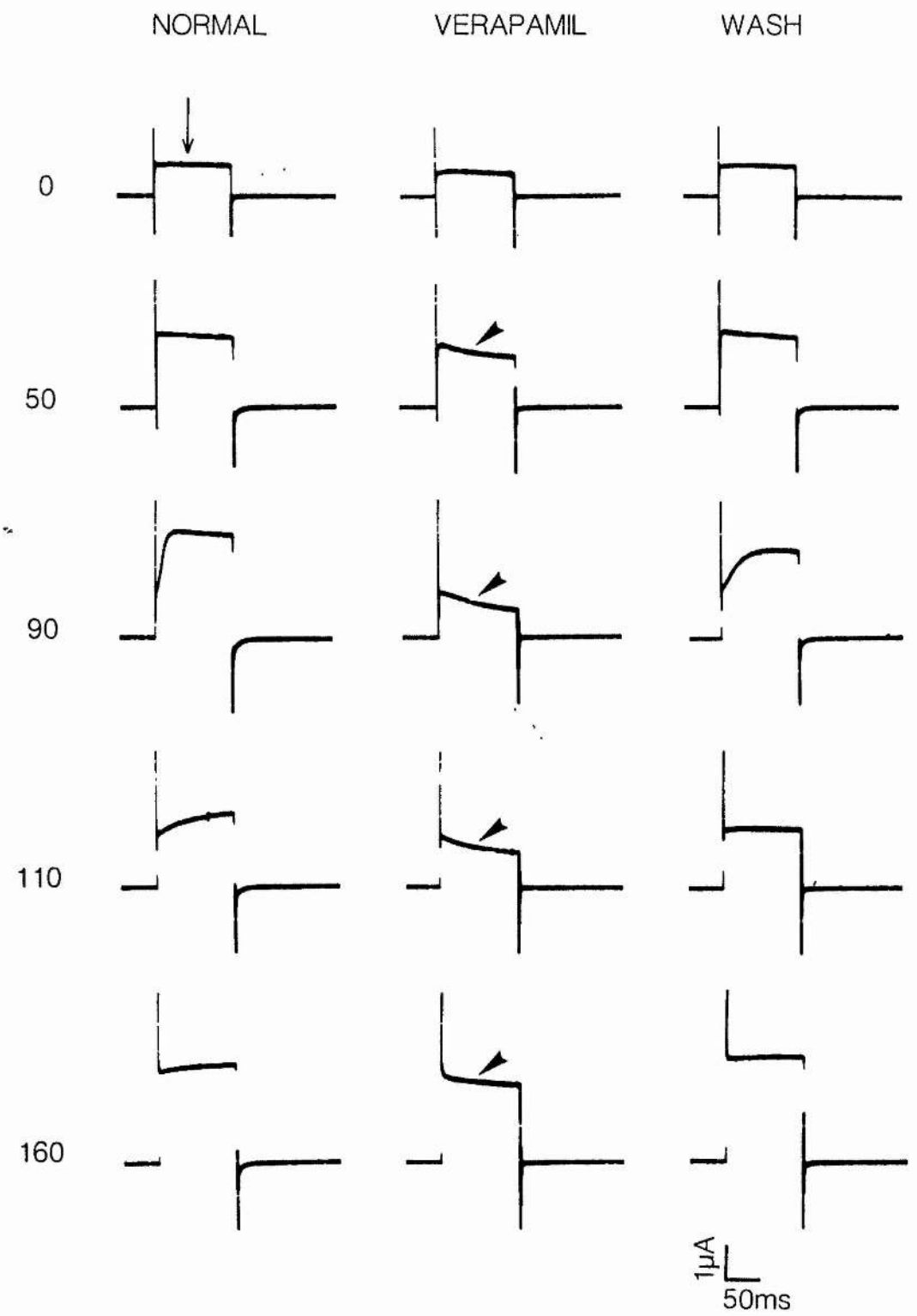
The effect of verapamil on the net outward current.

Selected current traces taken at command potentials of 0, +50, +90, +110 and +160mV.

Within 35mins. verapamil (50uM) caused an overall reduction in currents and increased the rate of inactivation of the remaining current (arrowheads) particularly between command potentials of 0 and +110mV. In addition, verapamil abolished the delayed current rise and reduced the associated currents. These effects were partially reversed after 20mins. wash with normal saline.

Holding potential: -70mV.

Arrow denotes position of current measurements: 50ms.


NORMAL
VERAPAMIL
WASH
0
50
90
110
160
1μA
50ms

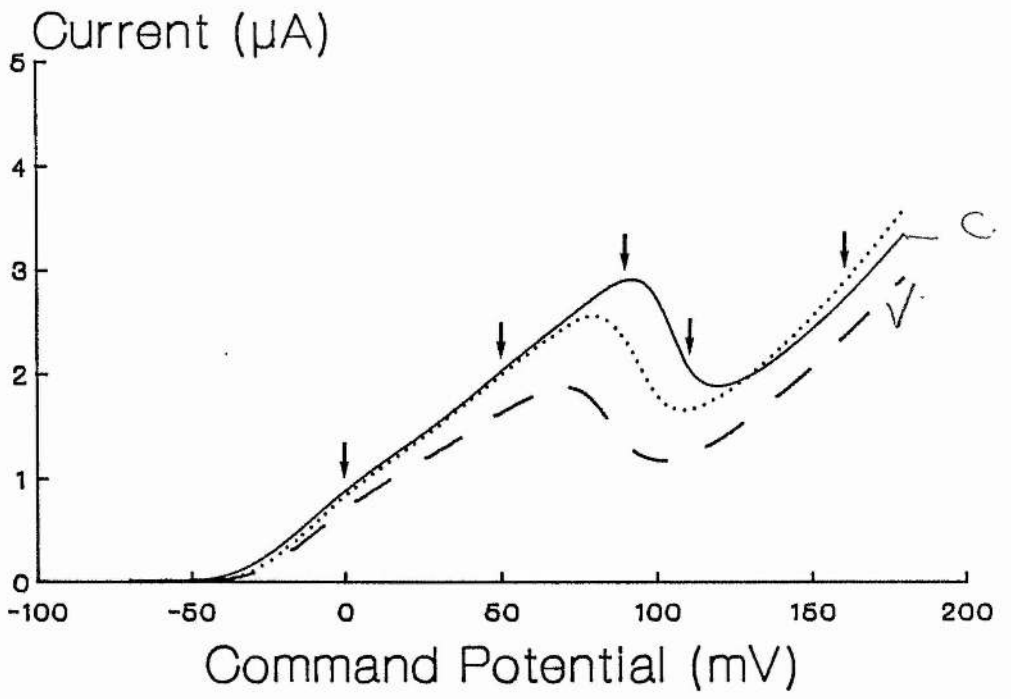


Fig. 3.2223 1B

The effect of verapamil on the I-V relationship.

Curves represent normal run (solid line) followed by 35mins. verapamil (50uM) (dashed line) and finally, after 20mins. wash with normal saline (dotted line).

Verapamil caused an overall reduction in the outward current and a shift in the hump by -20mV. Currents underlying the hump were reduced. These effects were partially reversed after washing with normal saline.

Arrows denote the position of selected current responses: 0, +50, +90, +110 and +160mV.

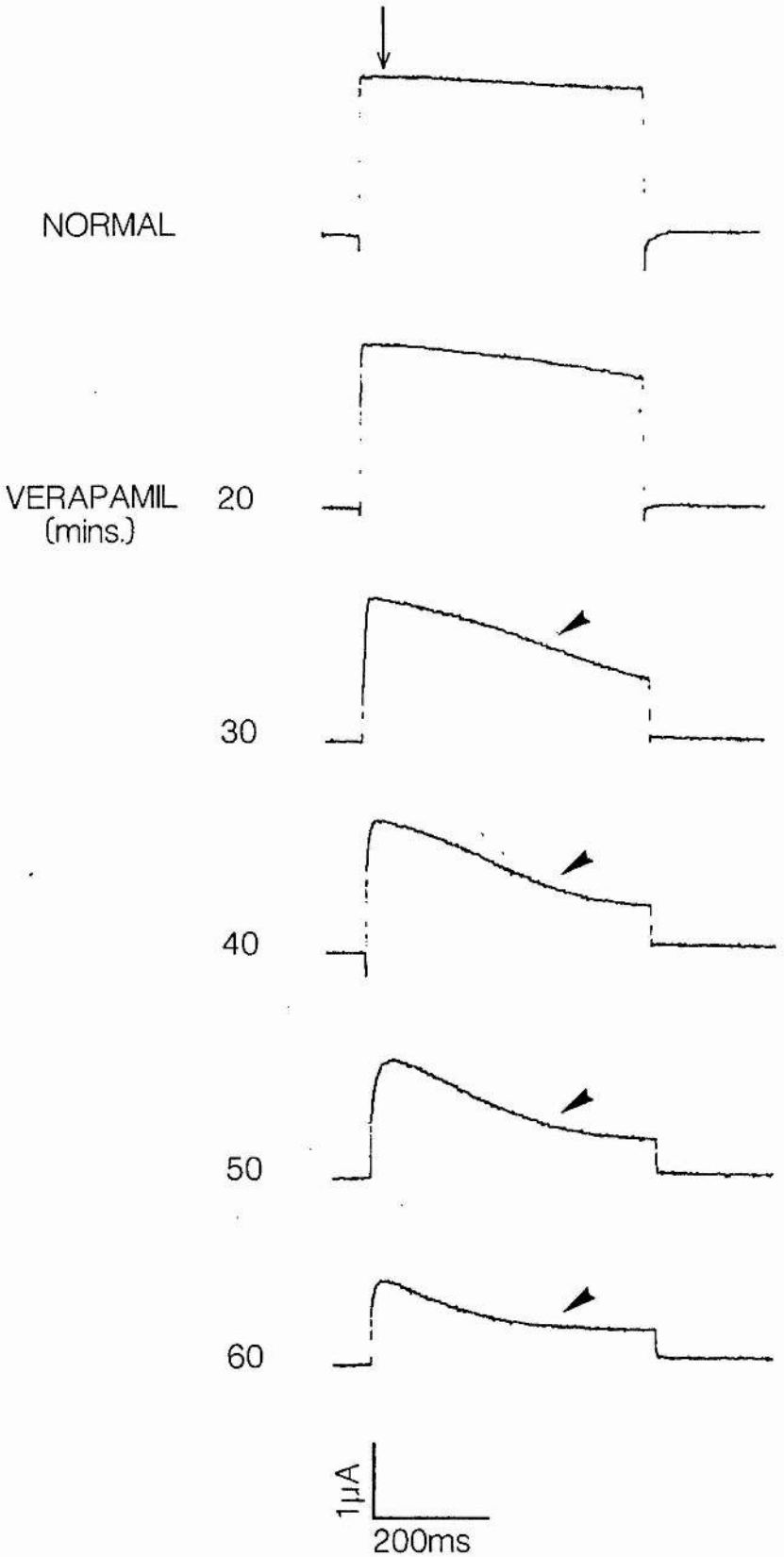
Fig. 3.2223 2A

The effect of prolonged exposure to verapamil on the net outward currents.

Selected current traces taken at command potentials of +50mV.

Prolonged exposure to verapamil (100μM) caused a time-dependent suppression of the outward currents. Furthermore, the inactivation caused by verapamil was more apparent later on in the command pulse (arrowheads).

Arrow denotes the position of current measurements: 50ms.


NORMAL
VERAPAMIL
(mins.)
20
30
40
50
60
1μA
200ms

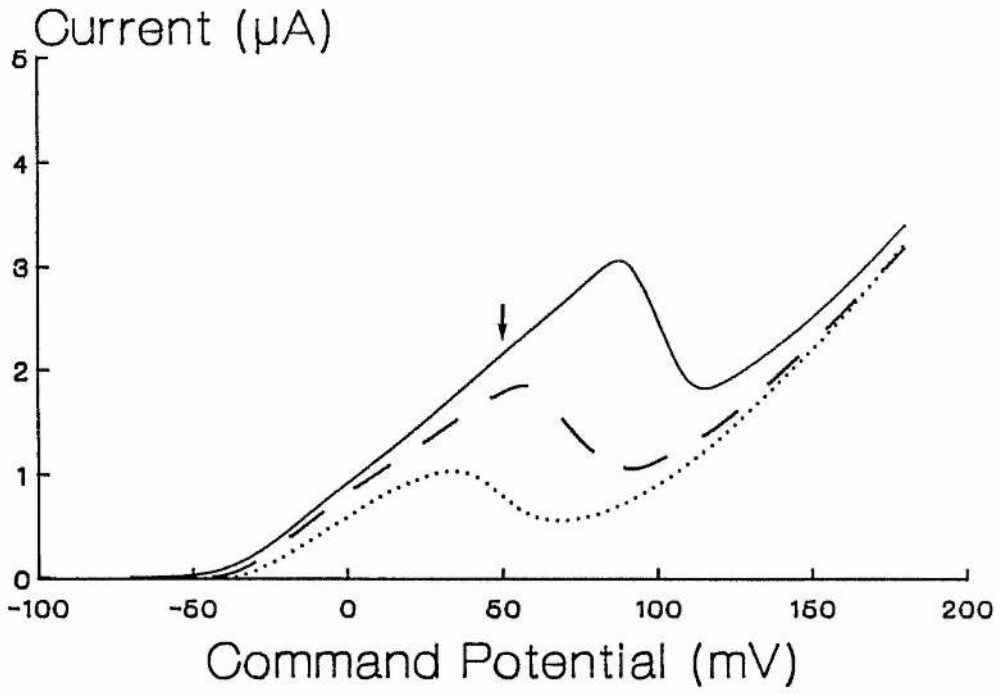


Fig 3.2223 2B

The effect of prolonged exposure to verapamil on the I-V relationship.

Curves represent normal run (solid line), 30mins. (dashed line) and 60mins. verapamil (100μM) (dotted line).

Prolonged exposure to verapamil caused a flattening of the curve and a more complete block of the hump, though a residual peak remained around +40mV.

Arrow denotes the position of selected current traces: +50mV.

20μM over 70mins. had little effect on the individual current responses (Fig. 3.2224 1A) or the corresponding I-V relationship (Fig. 3.2224 1B). There was a shift in the activation of the hump toward more negative potentials which may be due to the long duration of the impalement rather than an apamin effect. The magnitude of the negative conductance region remained unaffected.

Fig. 3.2224 1A

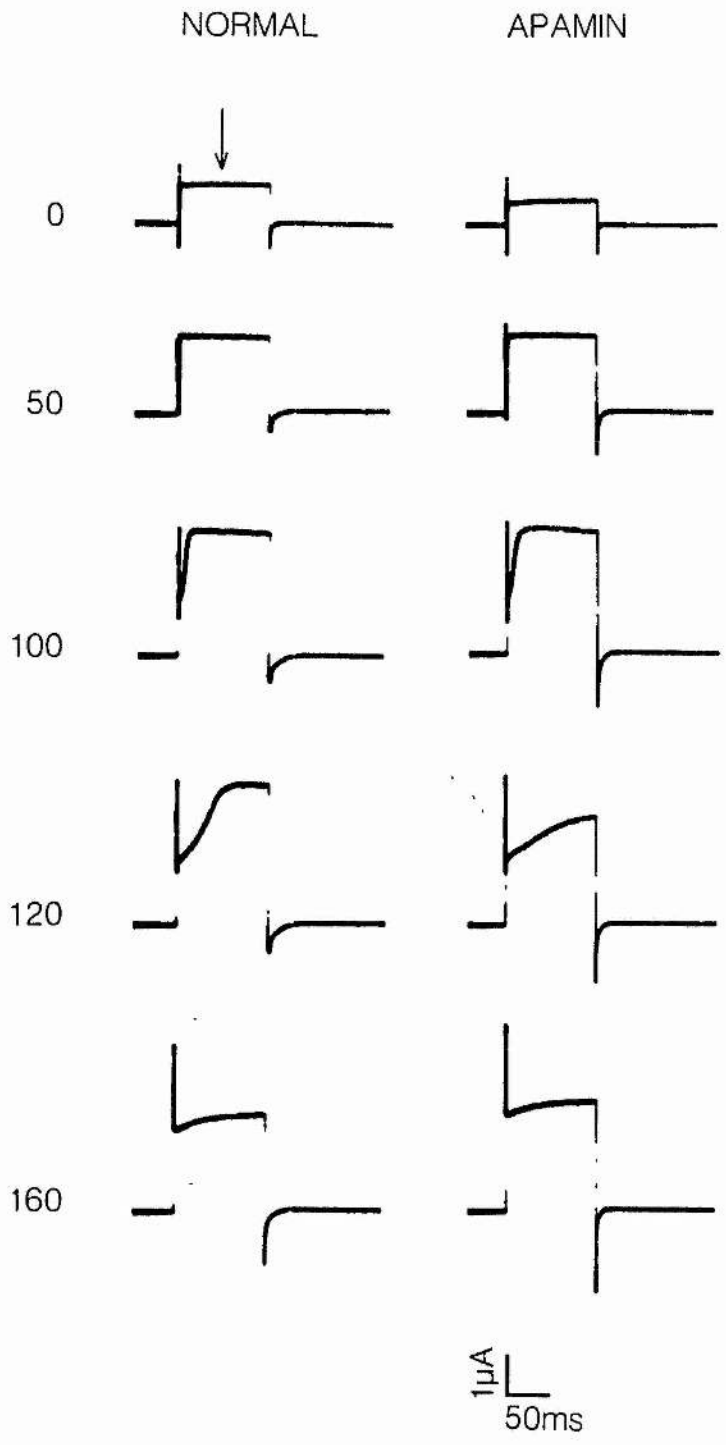
The effect of apamin on the net outward current.

Selected current responses taken at command potentials of 0, +50, +100, +120 and +160mV.

Within 70mins. apamin (total concentration 20uM) caused very little change in the current response though the delayed rise time was shifted toward more negative potentials.

Holding potential: -70mV

Arrow denotes the position of current measurements: 50ms.


NORMAL
APAMIN
0
50
100
120
160
1μA
50ms

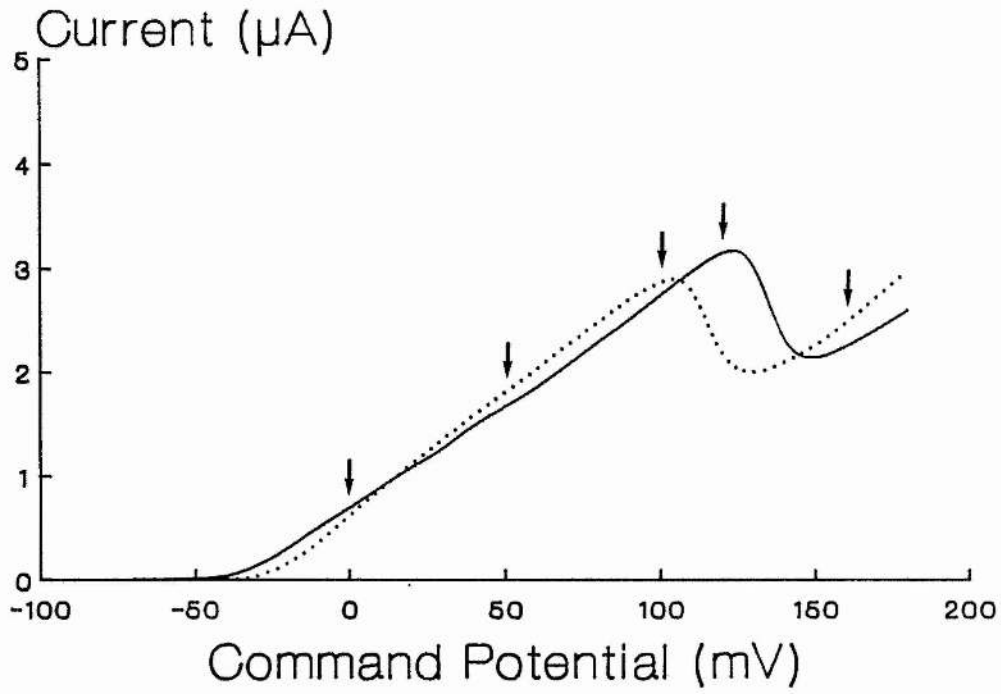


Fig. 3.2224 1B

The effect of apamin on the I-V relationship.

Curves represent normal run (solid line) and 70mins. apamin (total concentration 20uM) (dotted line).

During 70mins. apamin treatment there was a -20mV shift in the hump accompanied by a 0.3uA reduction in peak current.

Arrows denote the position of selected current responses: 0, +50, +100, +120 and +160mV.

3.223 Late delayed outward current.

Although a major portion of the outward current in cell 3 has been demonstrated to be calcium-dependent, when it is abolished by inorganic blockers a current component remains which increases in a non-linear manner as the command potentials become more positive (see Fig. 3. 2222 1B). Classically, TEA^+ has been reported to block a late outward current, I_K, in a variety of preparations (see section 1.221). The sensitivity of the outward currents to externally applied TEA^+ was subsequently investigated.

3.2231 TEA^+

Within 20mins., externally applied $TEA^+(Br^-)$ (50mM) completely abolished the current component with a delayed rise and caused an overall reduction of outward currents (Fig. 3.2231 1A). The hump in the I-V relationship was reduced to a residual peak around +30mV (Fig. 3.2231 1B). The effects were partially reversed after 20 mins. washout with normal saline. Exposure to TEA^+ for 10mins. showed that TEA^+ had further slowed the current rise between command potentials of +20mV and +50mV. There was also a reduction in the overall current response at potentials more positive than +50mV (Fig. 3.2231 2A). Within 30mins. the current component with a delayed rise was totally abolished and the overall current response further reduced. The hump in the I-V relationship was abolished and currents underlying the upper arm region were also reduced (Fig. 3.2231 2B). The proportion of total outward current affected by TEA^+ decreased as more positive potentials were reached. Compare the lower arm region of the curve (between -20mV and +70mV) with the upper arm region (between +120mV and +180mV). TEA^+ appeared to be less effective in blocking the outward current at more positive potentials.

3.2232 Cadmium and TEA^+.

By comparing the effects of TEA^+ with that of the inorganic blockers

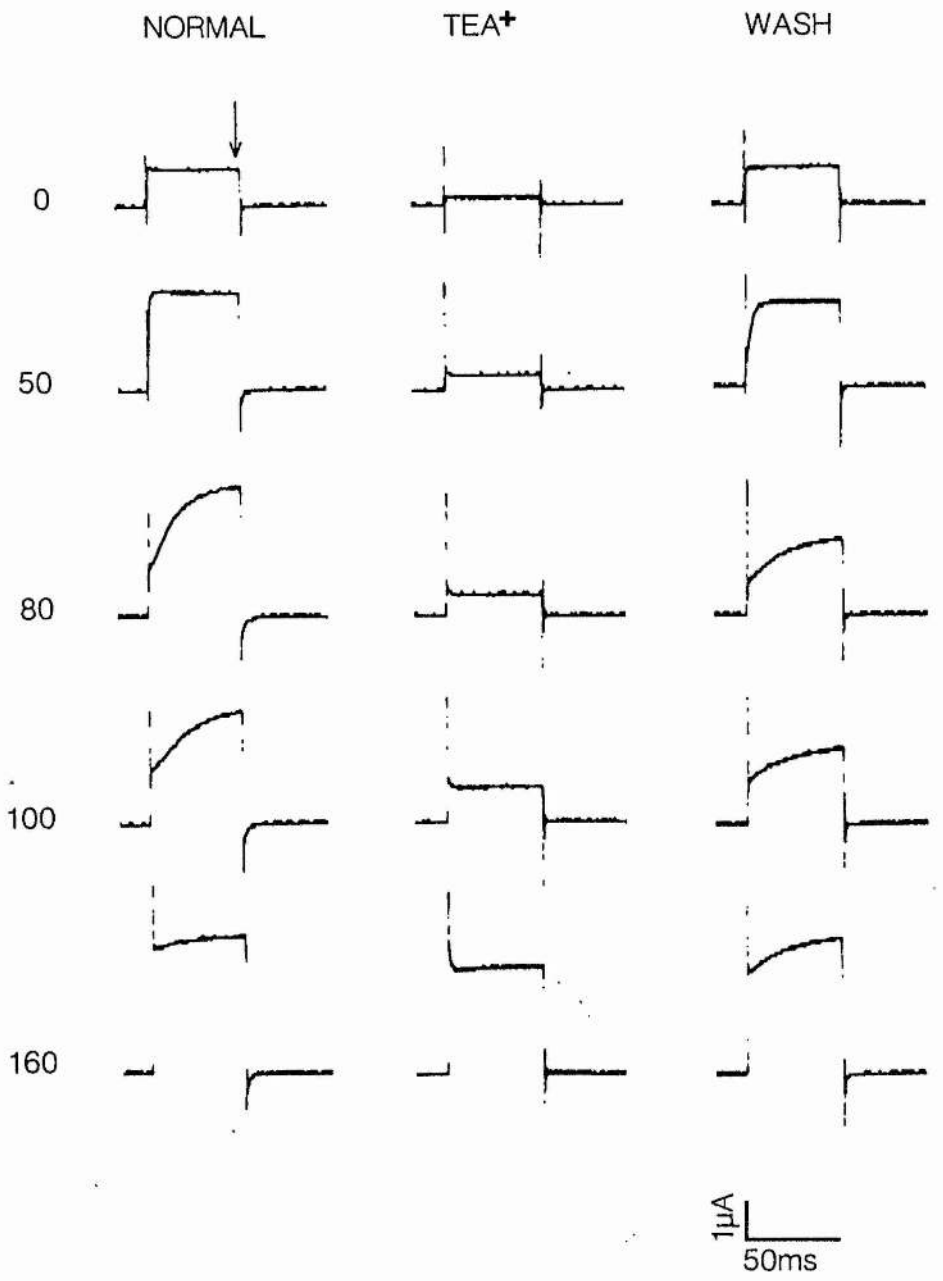


Fig. 3.2231 1A

The effect of TEA^+ on the net outward currents.

Selected current responses taken at command potentials of 0, +50, +80, +100 and +160mV.

Within 20mins. TEA^+ (50mM) had caused an overall reduction in currents and abolished the current underlying the delayed rise. These effects were partially reversed after 10mins. wash with normal saline.

Arrow denotes the position of current measurements: 50ms.

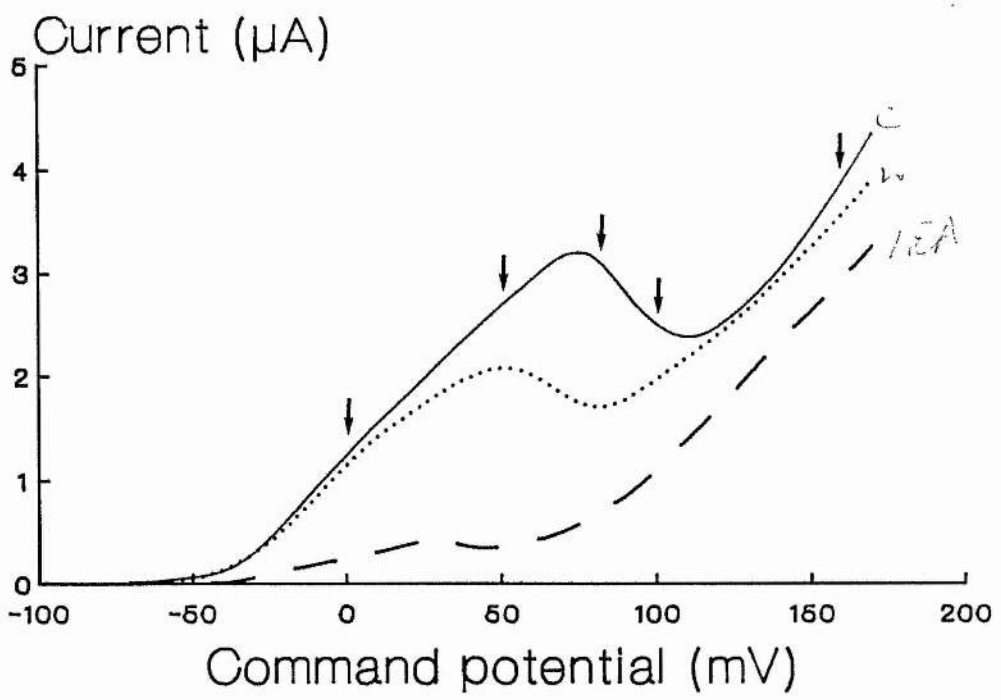


Fig. 3.2231 1B

The effect of TEA^+ on the I-V relationship.

Curves represent normal run (solid line) followed by 20mins. TEA^+ (50mM) (dashed line) and finally, 10mins. wash with normal saline (dotted line).

TEA^+-induced suppression of the I-V curve was partially restored after washing with normal saline

Arrows denote position of selected current responses: 0, +50, +80, +100 and +160mV.

Fig. 3.2231 2A

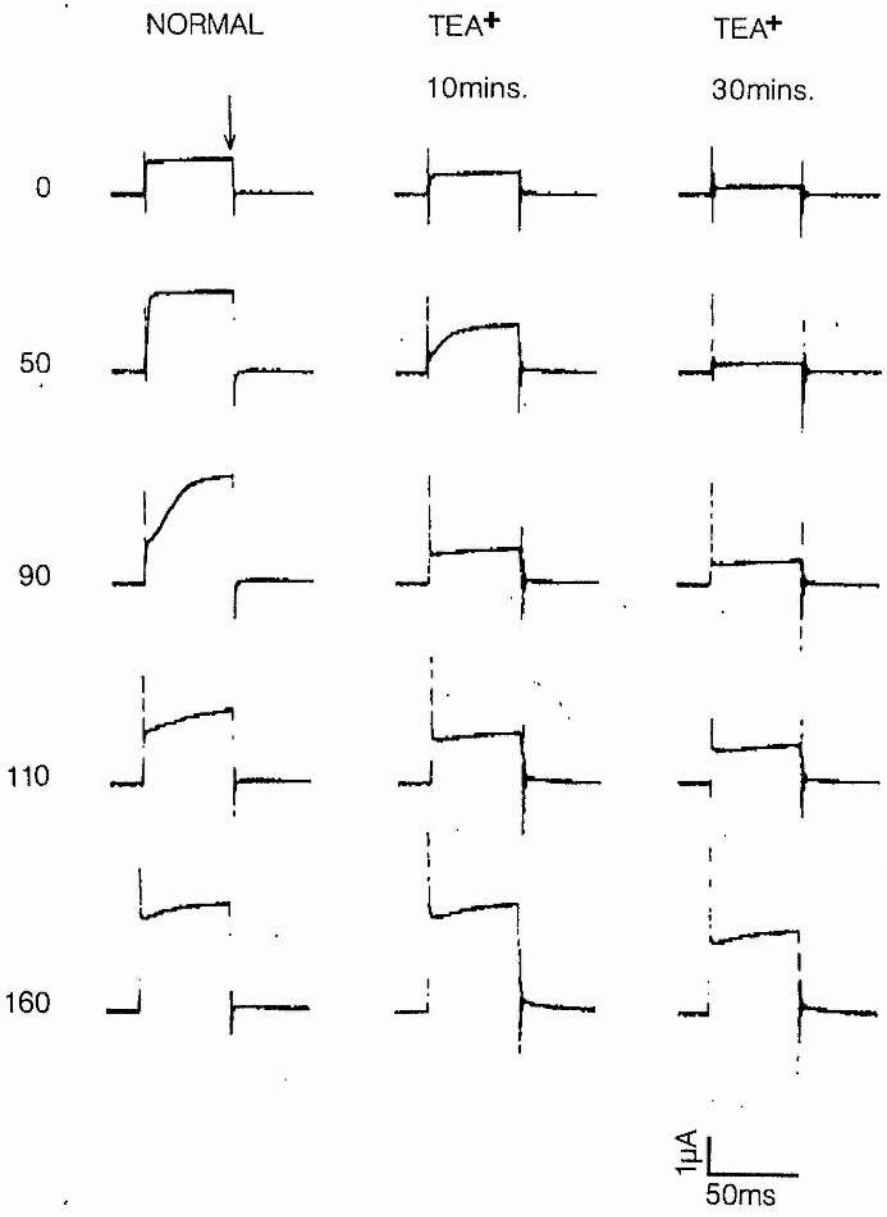
The effect of prolonged exposure to TEA^+ on the net outward currents.

Selected current responses taken at command potentials of 0, +50, +90, +110 and +160mV.

Within 10mins., TEA^+ (50mM) had increased the delay of the current rise between command potentials of +20 and +50mV and caused an overall reduction in the current response. After 30 mins. there was a further overall reduction and complete abolition of the current underlying the delayed rise. In addition, tail currents became more outward during drug treatment.

Holding potential: -70mV.

Arrow denotes the position of current measurements: 50ms.


NORMAL
TEA+
10mins.
TEA+
30mins.
0
50
90
110
160
1μA
50ms

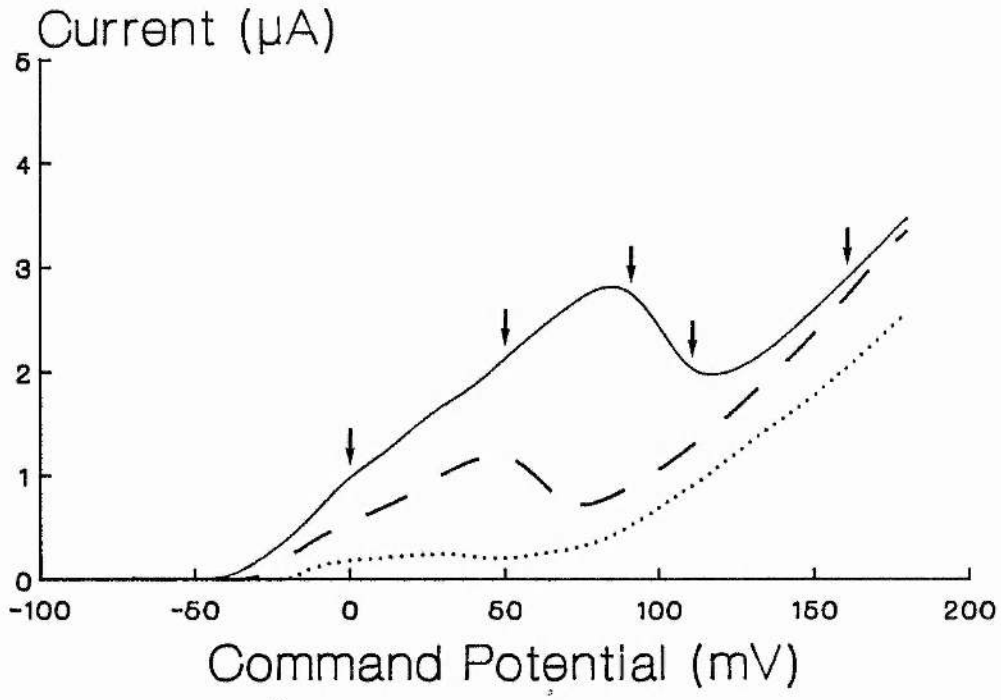


Fig. 3.2231 2B

The effect of prolonged exposure to TEA^+ on the I-V relatonship.

Curves represent normal run (solid line) followed by 10mins. (dashed line) and finally, 30mins. TEA^+ (50mM) (dotted line).

Within 30mins. TEA^+ caused a dramatic overall reduction in currents, especially between potentials of -20 and +110mV, and completely abolished the hump.

Arrows denote position of selected current responses: 0, +50, +90, +110 and +160mV.

Cd^{2+} and Mn^{2+} described above (see section 3.2222), TEA^{+} appeared to not only block the calcium-dependent current but also a further component of the outward currents. To determine the current components which were blocked by TEA^{+}, Cd^{2+} (1mM) was initially applied to the preparation in order to block the calcium-dependent current. As in previous experiments (Fig. 3.2222 1A), cadmium did abolish the current underlying the delayed rise time (Fig. 3.2232 1A) and the corresponding hump in the I-V relationship was reduced to a residual peak around a command potential of +90mV (Fig. 3.2232 1B). Note that in this experiment Cd^{2+} did not reduce currents in the upper arm of the curve . Subsequent application of TEA^{+} in the presence of Cd^{2+} (1mM) further depressed a portion of the outward current (Fig. 3.2232 1A) and caused a reduction in the I-V relationship which was almost parallel to that in the presence of Cd^{2+} (Fig. 3.2232 1B). These effects were completely reversed after 30mins. wash with normal saline. Even in the presence of Cd^{2+} and TEA^{+} there was still a residual current which steadily increased in a non-linear manner as command potentials became more positive. This suggests that the residual current was not solely a leakage current and may represent incomplete channel blockade, a current carried by another ion species or a Cd^{2+} and TEA^{+}-insensitive potassium current.

Fig. 3.2232 1A

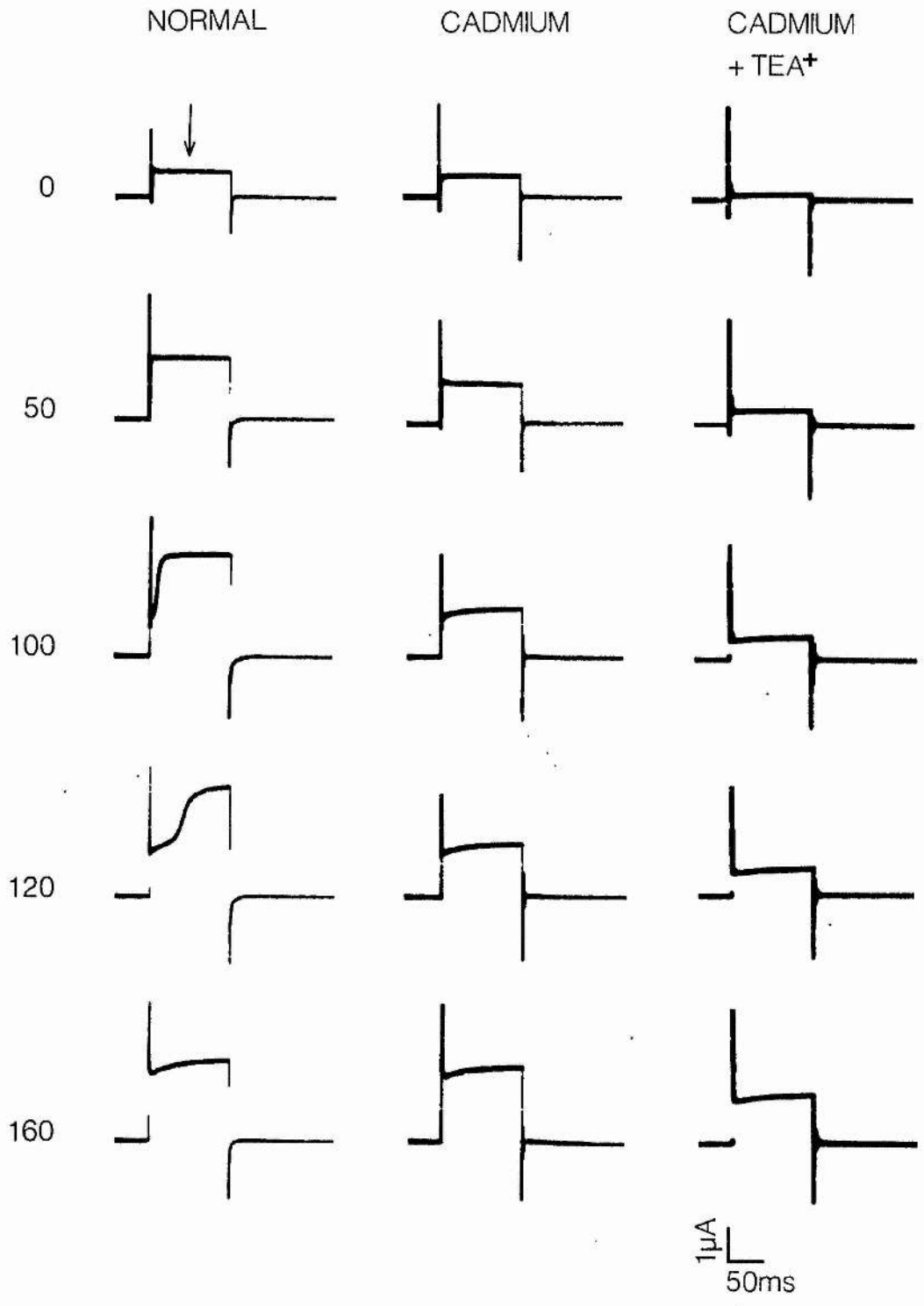
The effect of TEA^+, in the presence of cadmium ions, on the net outward currents.

Selected current responses taken at command potentials of 0, +50, +100, +120 and +160mV.

Within 15mins. Cd^{2+} (1mM) abolished the current underlying the delayed rise. Subsequent application of TEA^+ (25mM) further reduced a portion of the outward current. Even in the presence of both blockers an outward current persisted and increased with depolarisation.

Holding potential: -70mV.

Arrow denotes the position of current measurements: 50ms.


NORMAL
CADMIUM
CADMIUM
+ TEA+
0
50
100
120
160
1μA
50ms

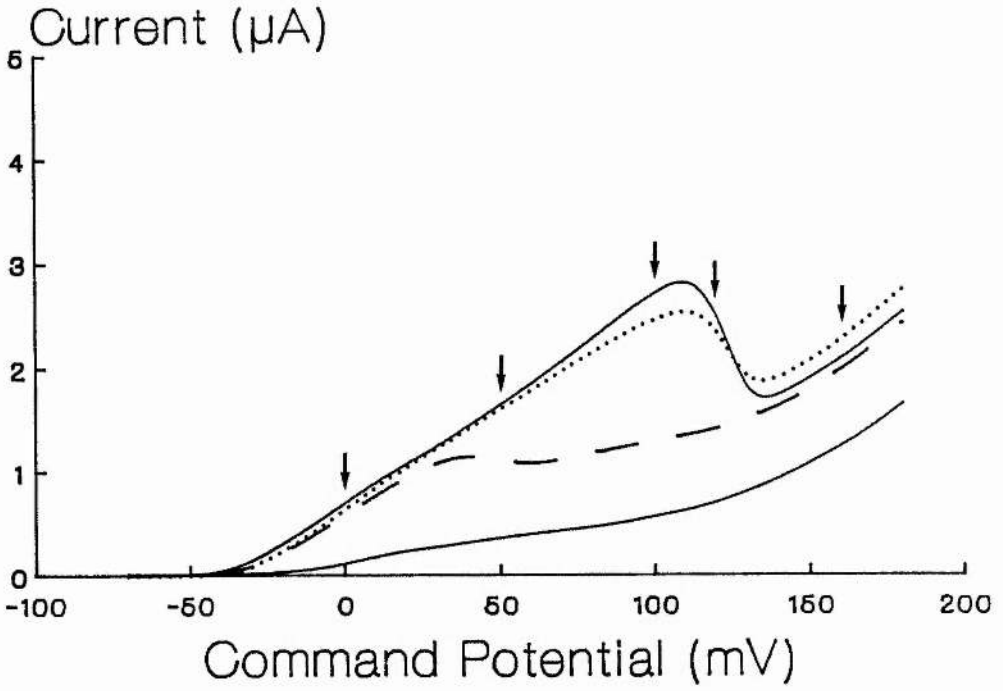


Fig. 3.2232 1B

The effect of TEA^+, in the presence of cadmium ions, on the I-V relationship.

Curves represent normal run (upper solid line) followed by 15mins. Cd^{2+} (1mM) (dashed line), then 5mins. TEA^+ (25mM) (lower solid line) and finally 30mins. wash with normal saline (dotted line).

Cd^{2+} reduced the hump to a residual peak around +40mV. Subsequent application of TEA^+ further reduced a portion of the outward current the remaining current increased in a non linear manner. These effects were reversed after washing with normal saline.

Arrows denote the position of selected current responses: 0, +50, +100, +120 and +160mV.

3.224 Low external chloride ion concentration.

Reducing the chloride content of the bathing saline to 20% of its normal value, i.e. from 235mM to 47mM, produced an overall reduction in outward currents and caused the delayed current rise to occur at more negative potentials (Fig. 3.224 1A). For the corresponding I-V relationship this resulted in a reduction in the peak current associated with the hump and a -50mV shift in the activation of the hump from 80mV to 30mV (Fig. 3.224 1B). Currents associated with the lower arm region were also reduced. In this experiment this represents a decrease in chord conductance (between 0 and +50mV) from 26uS to 20uS. In some but not all preparations the magnitude of the negative conductance region was marginally reduced in low chloride saline suggesting that the calcium-dependency of the region was affected in some way.

Fig. 3.224 1A

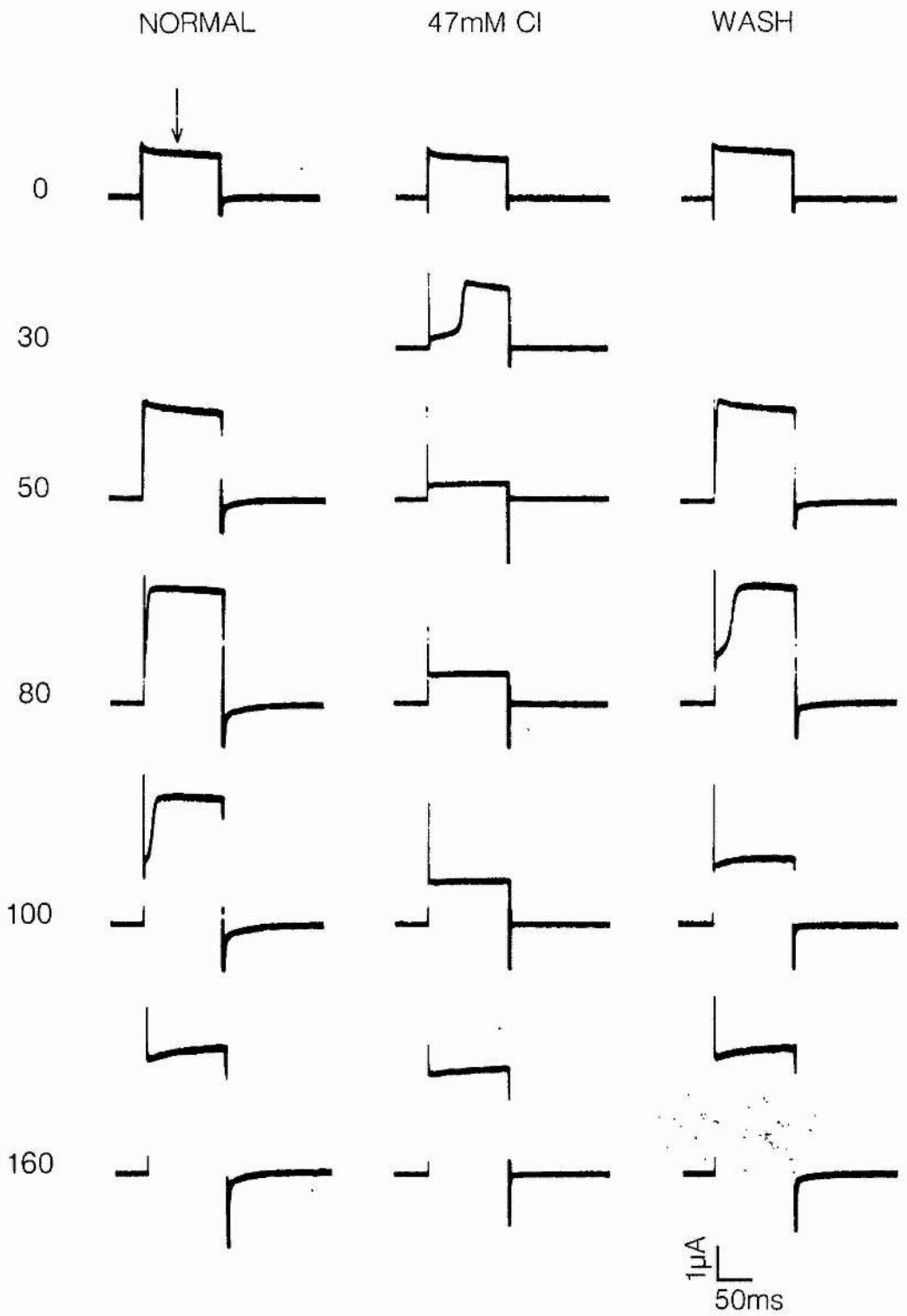
The effect of a five-fold reduction in external chloride ion concentration on the net outward currents.

Selected current responses taken at command potentials of 0, +30, +50, +80, +100 and +160mV.

Within 10mins. low Cl^- (47mM) saline caused an overall reduction in the current response with the delayed current rise shifted to more negative potentials. These effects were reversed after 15mins. wash with normal saline.

Holding potential: -70mV.

Arrow denotes the position of current measurements: 50ms.


NORMAL
47mM Cl
WASH
0
30
50
80
100
160
1μA
50ms

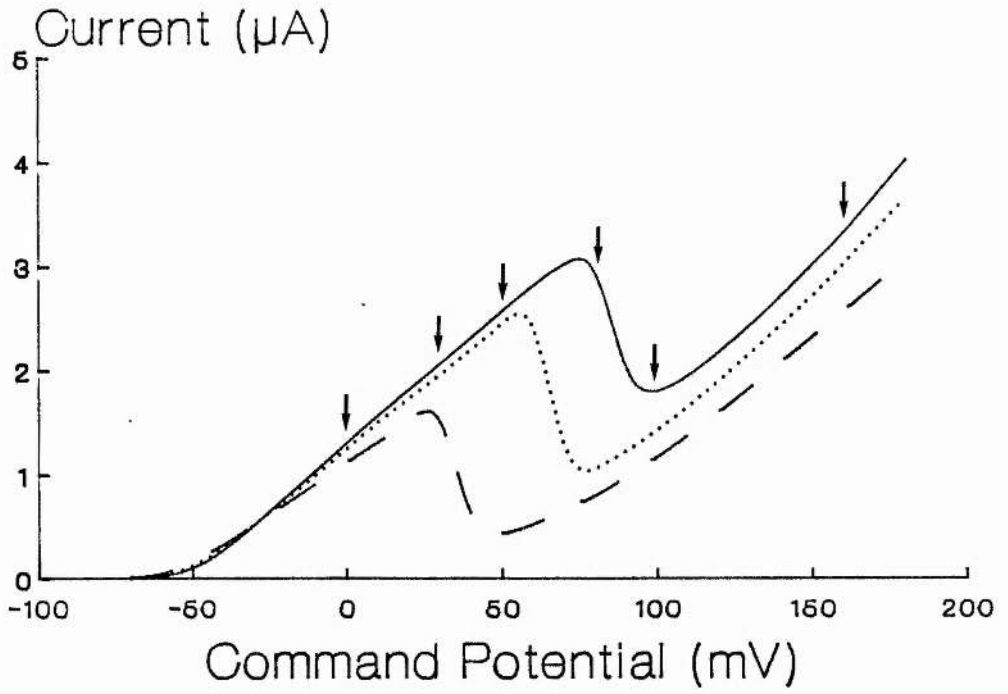


Fig. 3.224 1B

The effect of a five-fold reduction in external chloride ion concentration on the I-V relationship.

Curves represent normal run (solid line) followed by 10mins. Cl^- (47mM) saline (dashed line) and finally 15mins. wash with normal saline (dotted line).

Low Cl^- saline caused an overall reduction in currents with a -50mV shift in the hump.

Arrows denote the position of selected current responses: 0, +30, +50, +80, +100 and +160mV.

3.23 LONG-DURATION COMMAND PULSES.

After the pharmacology of outward currents evoked by short duration command pulses (100ms) had been investigated longer duration command pulses of up to three seconds were employed. For potentials less positive than +140mV the current which developed during the first second of the command pulse increased, then declined toward a steady-state plateau level, thereby producing a peak in the current response (Fig. 3.23 1A asterisk). The rising phase (activation) was much steeper than the falling phase. Whether the decline in the current response can be referred to as inactivation will be discussed in the following section. As the command potentials became more positive the rate of decline in current was increased and consequently the current took less time to reach a steady-state level (Fig. 3.23 1A arrowheads). Thus the falling phase of the current peak appeared to be voltage-dependent. The current reached a maximum around command potentials of +140mV, thereafter dropping down to the plateau level at more positive potentials. The current response between two and three seconds in the plateau region gradually increased as command potentials became successively more positive and did not appear to significantly decline during the course of the command pulse. For long duration command steps the tail currents were often all inward.

The I-V relationship showed a time- and voltage-dependency i.e. the magnitude and position of the hump in the N-shape depended on the time at which current measurements were taken into the command pulse (Fig. 3.23 1B). In addition, the N-shape I-V relationship was absent in currents associated with the plateau region instead they increased in a non-linear manner as command potentials became successively more positive.

The peak of the current response was reversibly abolished in cadmium (2mM) saline leaving the plateau region unaffected (Fig. 3.23

Fig. 3.23 1A

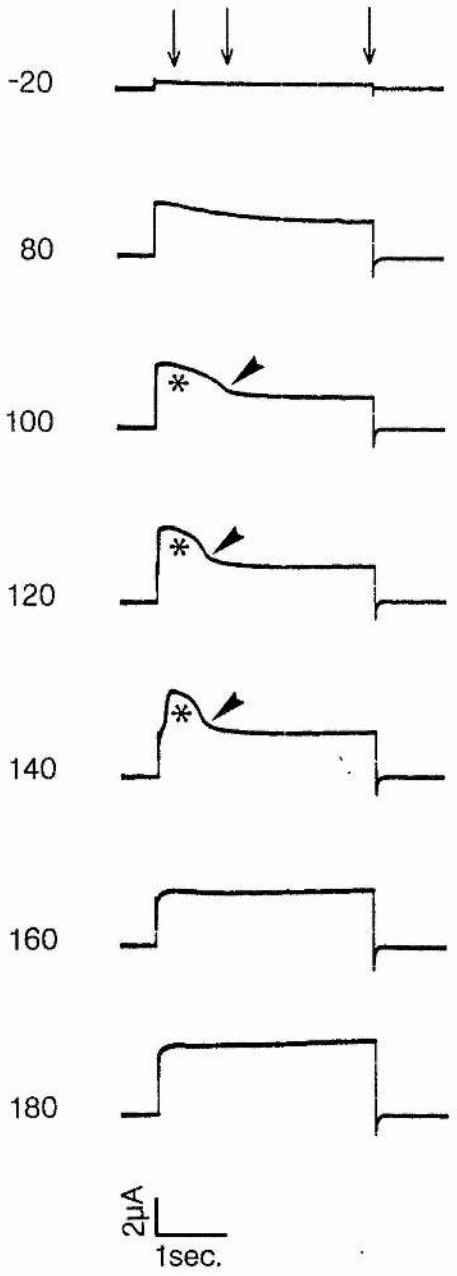
The effect of a three second command pulse on the net outward currents.

Selected current responses taken at command potentials of -20, +80, +100, +120, +140, +160 and +180mV.

At the command potentials between -20 and +140mV the current response during the first second increased to a maximum and thereafter fell to a lower steady-state giving rise to the current peak (asterisk). The rate of decline of the current peak increased with depolarisation between command potentials of +100 and +140mV (arrowheads). At potentials greater than 140mV the peak disappeared and the steady state current remained.

Holding potential: -70mV.

Arrows denote the position of current measurements: 250ms, 1 second and 3 seconds.


-20
80
100
120
140
160
180
2μA
1sec.

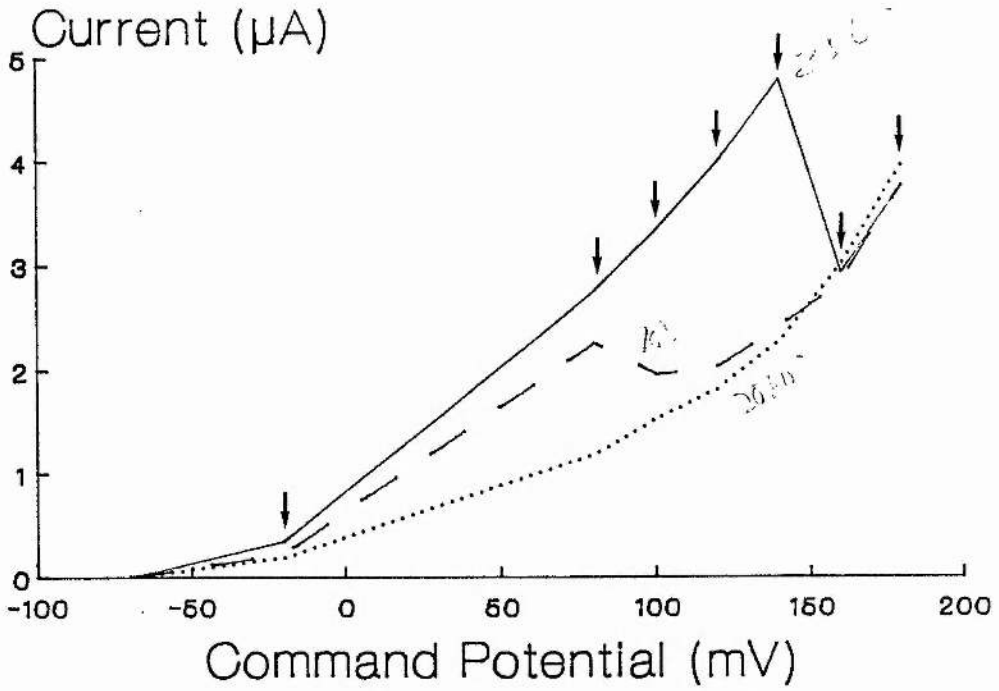


Fig. 3.23 1B

I-V relationship for net outward currents evoked by a three second command pulse.

Curves represent current measurements taken at 250ms (solid line), 1 second (dashed line) and 3 seconds (dotted line). Co-ordinates were joined by straight lines as there were insufficient data to achieve a satisfactory curve of best fit.

In this experiment a maximum peak current response of 4.8μA was measured at 250ms, at a command potential of +140mV. Current measurements taken after 250ms in the command pulse caused flattening of the N-shape curve which completely disappeared for measurements taken later than 1.5 seconds.

Arrows denote the position of selected current traces; -20, +80, +100, +120, +140, +160 and +180mV.

2). The current remaining after Cd^{2+} treatment i.e. the plateau region appeared analogous to the Cd^{2+}- and Mn^{2+}-insensitive currents observed with short duration command pulses (see Figs. 3.2222 1A and 2A). Preliminary results indicate that when a three second pulse to a potential evoking maximal current activation was repeated at 0.1Hz, within two minutes the rise time of successive responses decreased until the current peak disappeared altogether (not shown). After a period of 10-15mins. resting the current response returned to normal. A complete run of 25 positive command steps from the holding potential was not possible with long duration command pulses as this led to a decline in the current response with the abolition of the current peak.

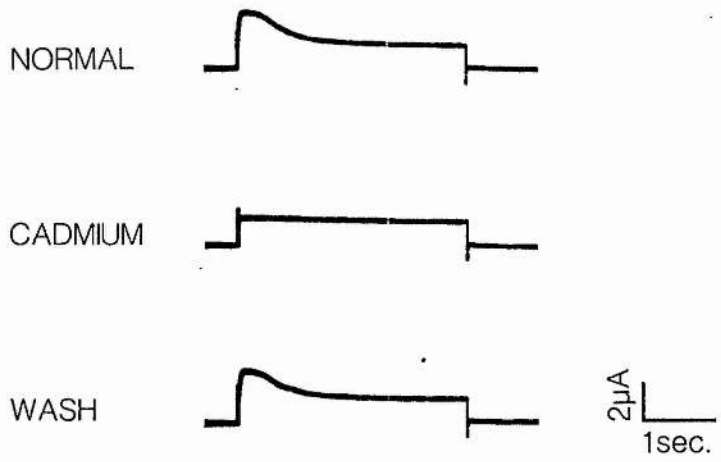


Fig. 3.23 2

The effect of cadmium on the net outward currents evoked by a three second command pulse.

Selected current responses from a three second command pulse to +100mV.

Within 10mins. externally applied Cd^{2+} (2mM) was able to abolish the current peak without significantly affecting the plateau region. The current peak was restored after 30mins. wash with normal saline.

Holding potential: -70mV.

3.24 TAIL CURRENTS.

3.241 Single command pulse regime

Command pulse durations of 50ms or greater were employed in characterising of the net outward currents described above (section 3.22) which, when stepping positive from a holding potential of -70mV, produced inward tail currents. In order to study the tail currents using a single command pulse, short duration command pulses between 10ms and 50ms were employed.

3.2411 Command pulse duration dependency.

Command pulse duration was varied between 10ms and 50ms to examine the effect of its duration upon the polarity and magnitude of the tail currents. Current measurements were taken at 5ms and 10ms after the end of command pulses. Selected tail current responses are shown in Fig. 3.2411 1A. There were two trends within the tail current response:

i) For a given command potential the tail currents became inward as the command pulse duration increased.

ii) For pulse durations greater than 20ms, as command steps became more positive, the outward tail currents became more inward (Fig. 3.2411 1A arrowhead) between potentials of +30 and +70mV, then once again outward and, finally inward (asterisk Fig 3.2411 1A) giving rise to an M-shaped I-V relationship (Figs 3.2411 1Bii, and iii).

Increasing the command pulse duration for a given command potential clearly showed that the currents became more inward. The inward current dip which peaked around +60mV, was more pronounced for measurements taken at 5ms. Note that the tail currents from short command pulses of 10ms, were outward for all command potentials between 0 and +160mV.

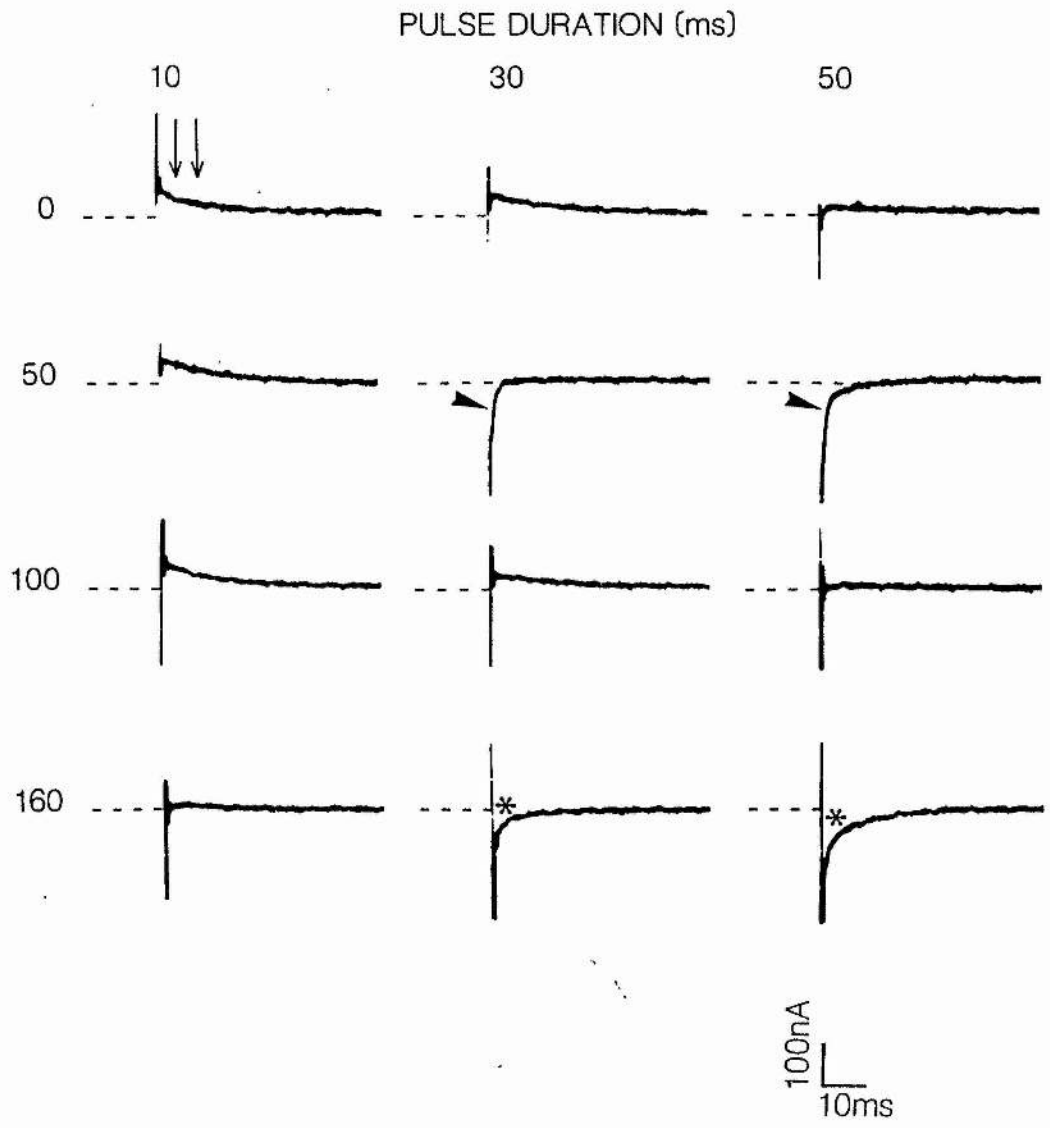


Fig. 3.2411 1A

The effect of increasing the command pulse duration on the tail current response.

Selected tail current responses from command potentials of 0, +50, +100 and +160mV.

As the command pulse duration was increased the tail current response for a given command potential became more inward. A 10ms command pulse duration produced tail currents which were all outward. Longer duration command pulses had a fast initial component at +50mV (arrowheads) and a much slower decaying inward current at +160mV (asterisk).

Holding potential: -60mV.

Arrows denote the position of current measurements: 5 and 10ms.

Fig. 3.2411 1B

The effect of increasing the command pulse duration on the tail current I-V relationship.

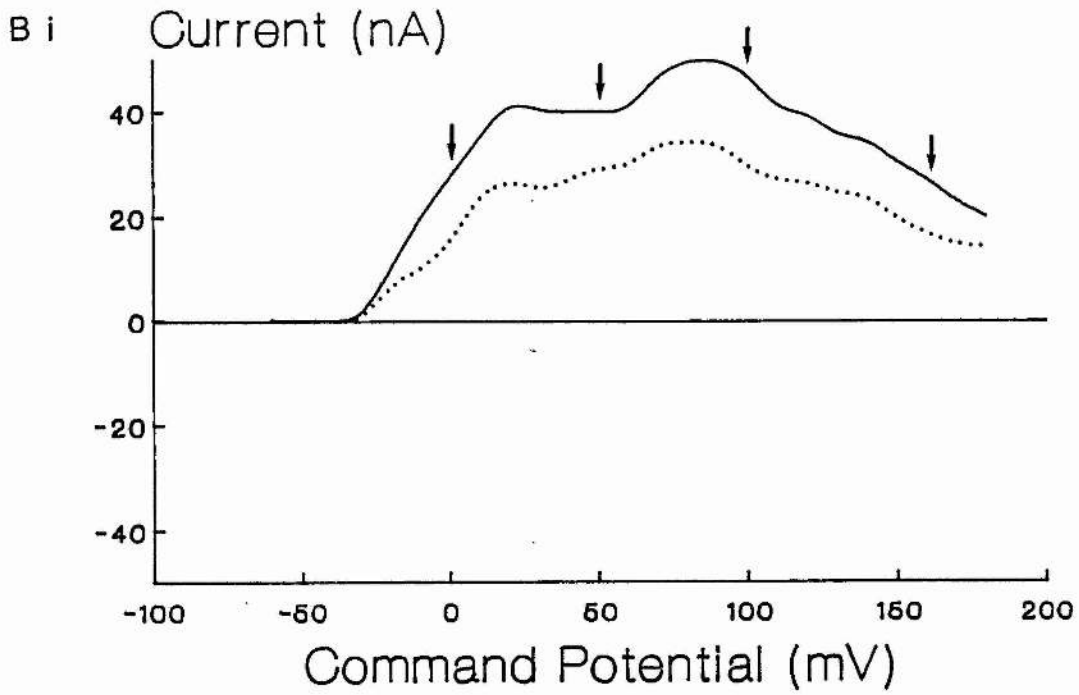
Current measurements taken at 5ms (solid line) and 10ms (dotted line) after repolarisation to -60mV.

i. Command pulse duration of 10ms. All the tail currents were outward. There was a small inward dip around +50mV for current measurements taken at 5ms. The tail currents became less outward at command potentials more positive than -90mV.

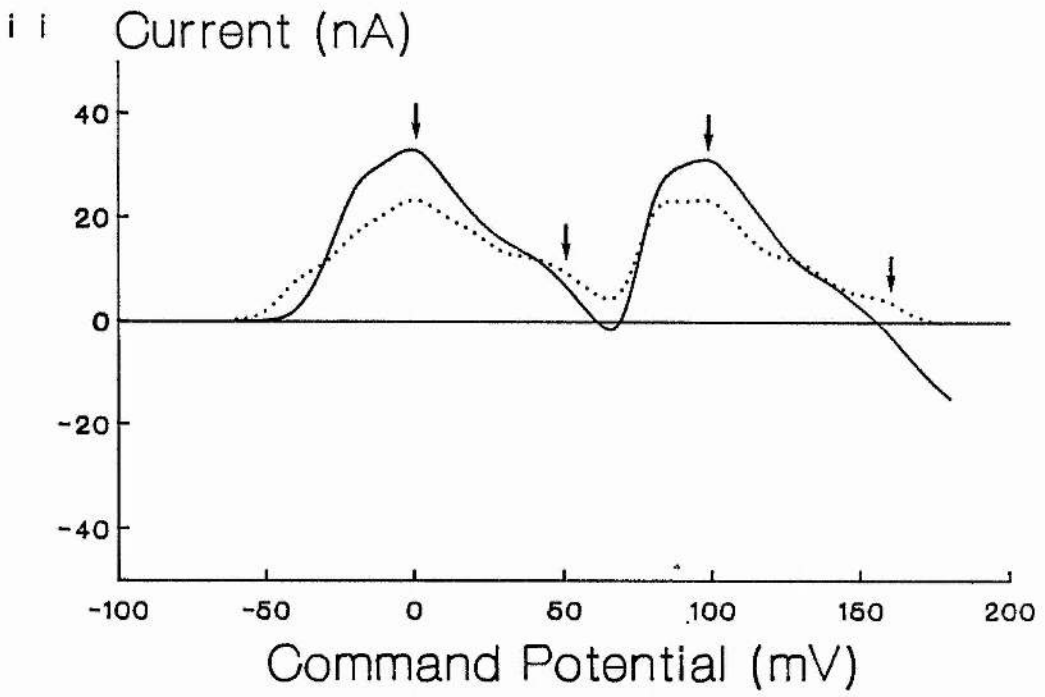
ii. Command pulse duration of 30ms. There was a pronounced dip in the curve around +70mV where the tail currents became transiently less outward giving the I-V relationship a definite M-shape.

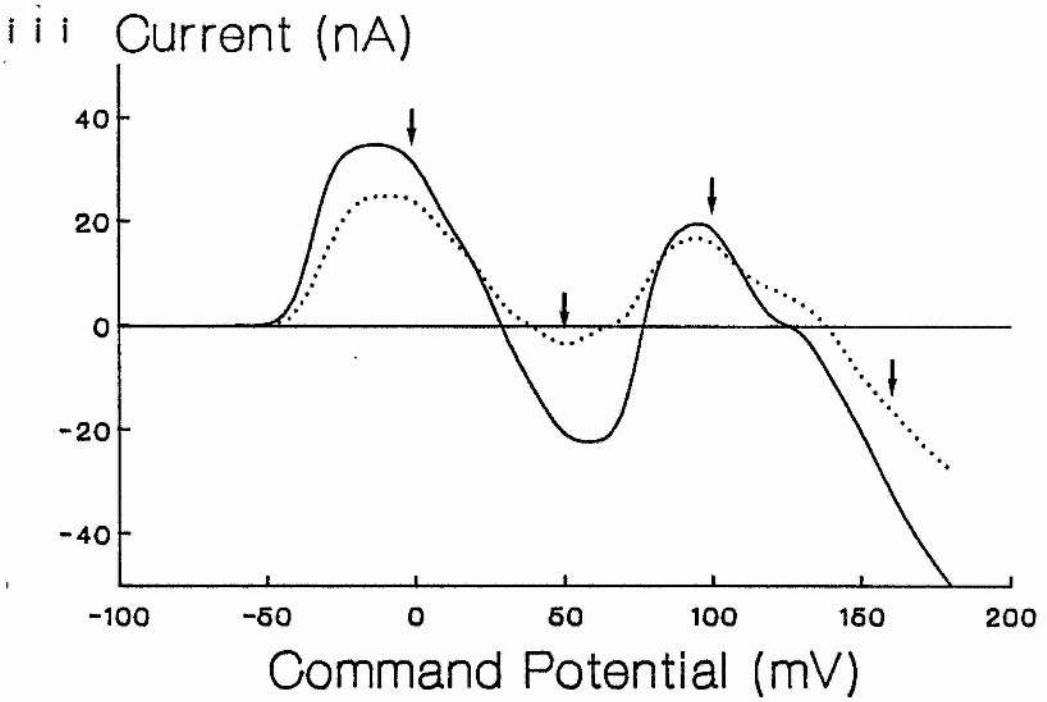
iii. Command pulse duration of 50ms. The current dip was more pronounced becoming transiently inward between potentials of +30mV and +70mV, peaking around +60mV. As the command potential increased the currents became markedly inward.

Current measurements taken at 5ms were more sensitive to increasing command pulse duration than measurements taken at 10ms, in the tail current relaxation.



dif due K ?
(14 ?)


i i
Current (nA)
40
20
0
-20
-40
-100
-50
0
50
100
150
200
Command Potential (mV)


i i i
Current (nA)
40
20
0
-20
-40
-100
-50
0
50
100
150
200
Command Potential (mV)

Figs. 3.2411 2i & ii show graphs of current measurements (taken at 5ms and 10ms respectively) at a range of command pulse durations for a given command step to 0, +50 and +100mV. Increasing the command pulse duration caused all of the tail currrents to become inward at more positive potentials. Tail current measurements taken earlier on in the response i.e. at 5ms, were affected more than those taken at 10ms though the trend was similar. Furthermore, measurements from command potentials of +50mV were particularly sensitive to the pulse duration and rapidly became inward at more positive potentials.

Fig. 3.2411 2

The effect of command pulse duration on tail currents from selected command potentials.

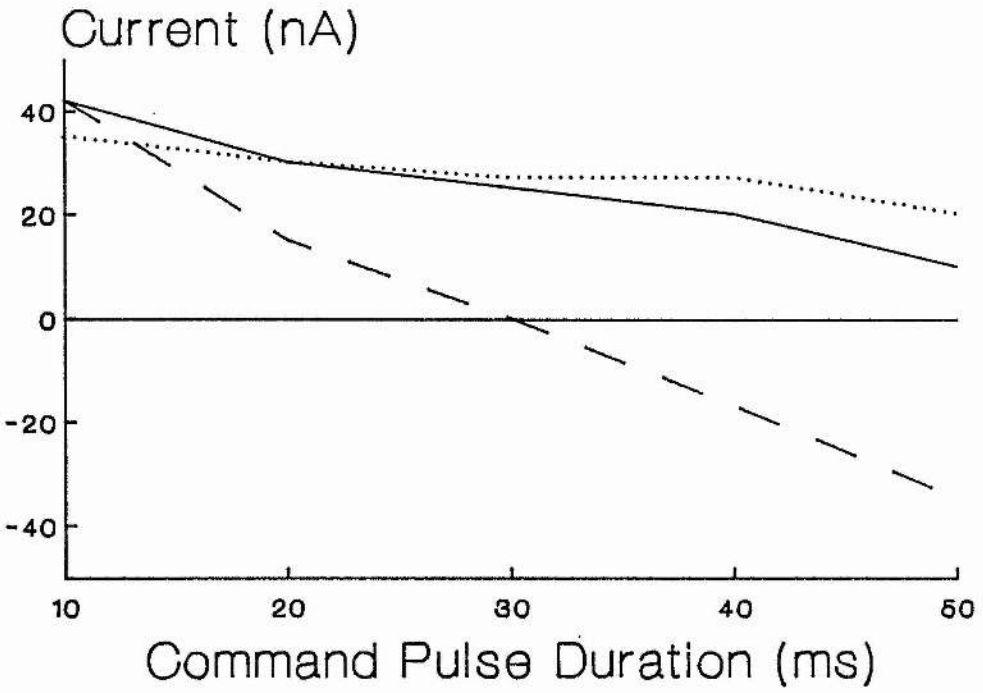
Lines represent command potentials of 0 (dotted line), +50 (dashed line) and +100mV (solid line).

Current measurements taken at i. 5ms and ii. 10ms.

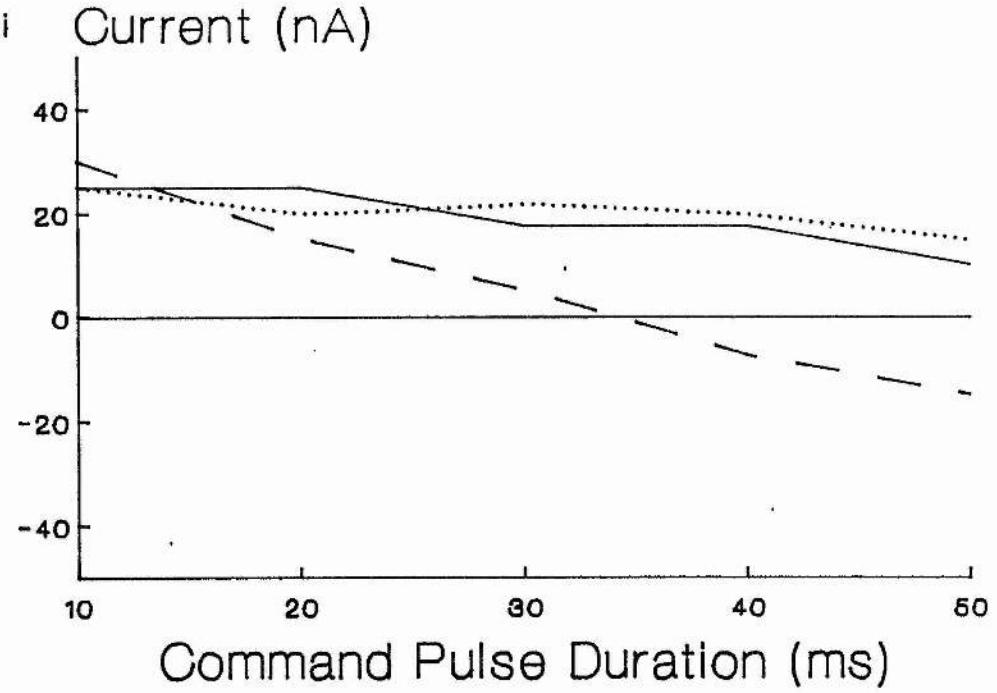
As the command pulse duration was increased the tail currents became less outward for all potentials shown. Tail currents from a command step to +50mV were particularly sensitive to increasing pulse duration and rapidly became inward. The trend was similar, but not so marked, for tail current measurements taken at 10ms compared to those taken at 5ms.

7 2.

Bi


Current (nA)
40
20
0
-20
-40
10
20
30
40
50
Command Pulse Duration (ms)

ii


Current (nA)
40
20
0
-20
-40
10
20
30
40
50
Command Pulse Duration (ms)

3.242 Double command pulse regime

In the previous section (3.241) tail currents were measured after single depolarising command steps from the holding potential. In order to study tail currents in a more controlled manner a double commmand pulse regime (Fig. 2.251 C) was adopted whereby a standard depolarising pre-pulse (pulse (I)) was employed to open a given population of channels. These open channels take a finite time to close and, when the membrane was stepped to a new potential (Pulse (II)), tail currents flowed. The direction and magnitude of the current flow can be given by the equation:

$$I_t = g(V_C - V_{Rev}) \qquad \text{Eq. 3.242 1}$$

Where I_t is the tail current, g is the conductance, V_C is the command potential and V_{Rev} is the reversal potential. The provisional tail current data are presented in the following sections.

3.2421 Pulse (I) magnitude dependency.

Fig. 3.2421 1A shows selected tail current responses from a pulse (I) duration of 20ms to command potentials of 0mV, +50mV and +100mV. Between pulse (II) potentials of -120 and -80mV a small, steady inward tail current flowed, and thereafter an outward tail current developed. Within the initial 10ms the outward tail currents underwent rapid decline followed by a slower decline which was not complete within the 50ms pulse (II) duration. For pulse (I) +50mV the tail currents had an inward component which occurred immediately after the capacitative transient. Increasing the pulse (I) magnitude resulted in an increase in outward tail currents. Assuming that a greater depolarisation during pulse (I) would open more ion channels this increase in tail current response was expected.

The I-V relationships for the tail currents showed a marked outward rectification between pulse (II) command potentials of -60mV and -80mV, depending on the recording parmeters (Figs. 3.2421 1Bi &

ii). Current measurements taken at 10ms had more negative reversal potentials than those taken at 5ms.

Fig. 3.2421 1A

The effect of pulse (I) magnitude on the tail current response.

Selected tail current responses taken at pulse (II) potentials of -110, -90, -70, -50 amd -30mV. Standard 20ms pulse (I) duration.

Pulse (I) current response is not shown and the dotted line indicates the steady-state current level at the holding potential.

The tail currents were little affected by the pulse (I) magnitude at pulse (II) command potentials below -70mV and remained inward. A pulse (I) magnitude of +50mV evoked tail currents with an initial fast inward component (large arrowhead). At pulse (I) command potentials greater than -70mV the tail currents became outward. Larger pulse (I) magnitudes produced larger outward tail currents. The outward tail currents had an initial rapid (small arrowheads) followed by a slow inactivation phase.

Holding potential: -70mV.

Arrows denote the position of current measurements: 5 and 10ms.

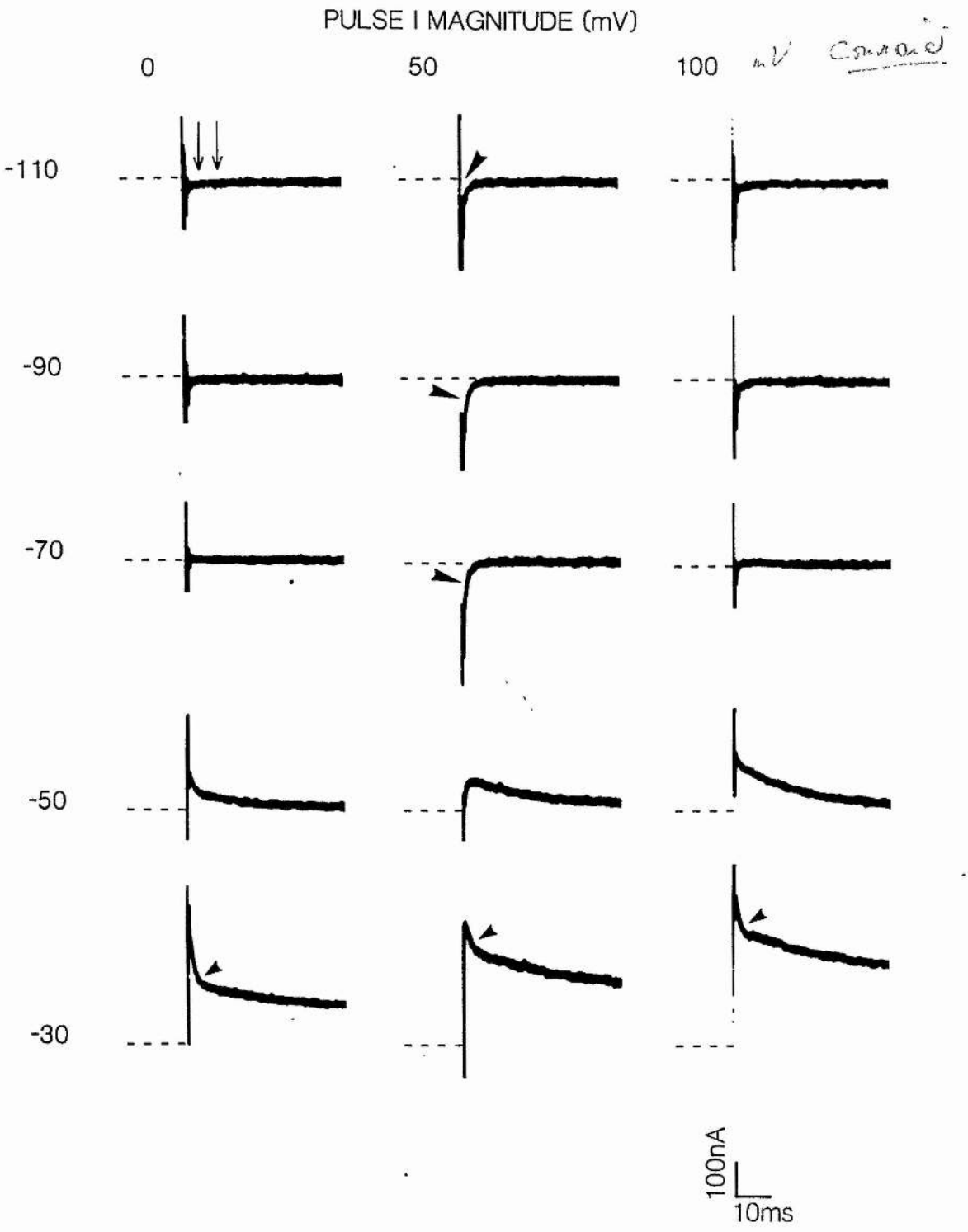

PULSE I MAGNITUDE (mV)
0
50
100
-110
-90
-70
-50
-30
100nA
10ms

Fig. 3.2421 1B

The effect of pulse (I) magnitude on the tail current I-V relationship.

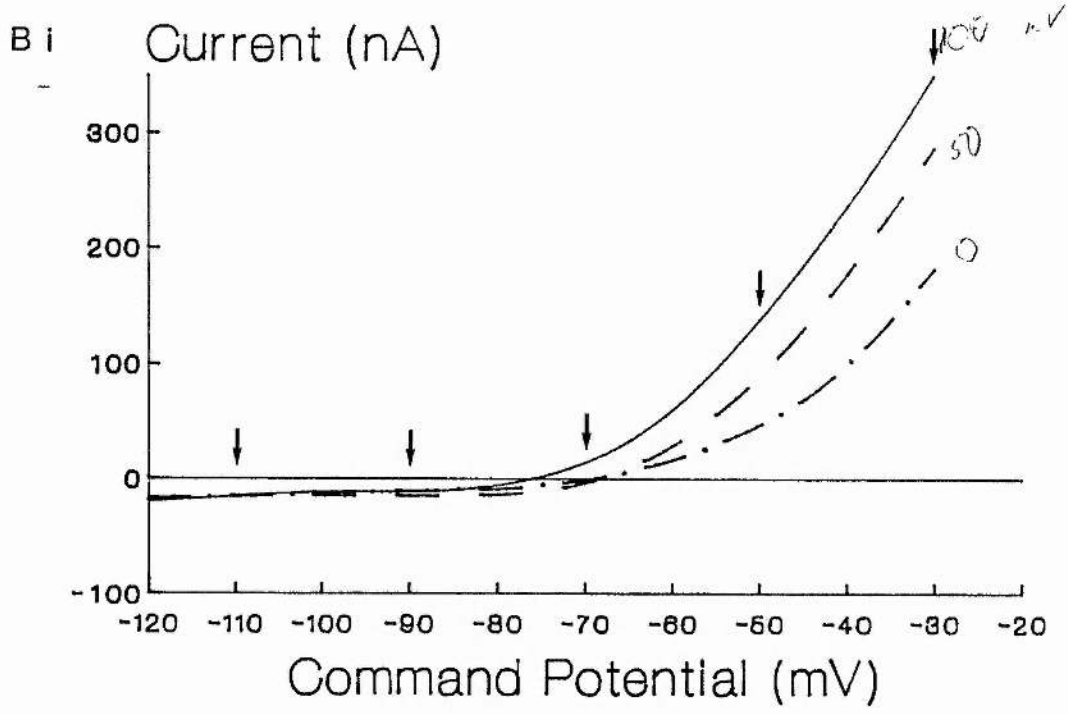
Curves represent pulse (I) magnitudes of 0mV (dot-dashed line), 50mV (dashed line) and 100mV (solid line).

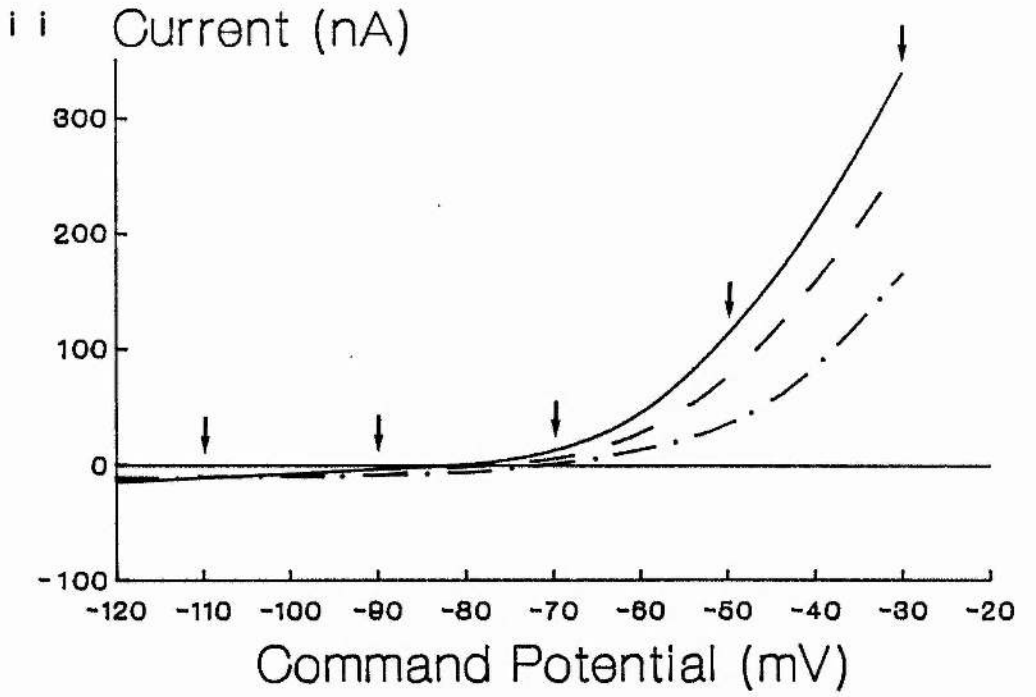
The tail currents showed marked outward rectification between command potentials of -60 and -80mV.

i. Tail current measurements taken at 5ms. Tail currents from pulse (I) magnitudes of 0, +50 amd +100 reversed at -69, -69 and -77mV respectively.

ii. Tail current measurements taken at 10ms. Tail currents from pulse (I) magnitudes of 0, +50 and +100 reversed at -71, -77 and -81mV respectively.

As the pulse (I) magnitude increased the tail current reversal potentials became more negative and the magnitude of the outward tail currents increased.


B i
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)


i i
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)

3.2422 Pulse (I) duration dependency.

Pulse (I) command step to 0mV.

For a pulse (I) command step to 0mV increasing the pulse (I) duration from 10ms to 50ms had very litte effect on the tail current response (Fig. 3.2422 1A). The corresponding I-V relationship for measurements taken at 5ms and 10ms showed that increasing the pulse (I) duration resulted in the reversal potential becoming more positive (Figs. 3.2422 1Bi and ii). Note that as the pulse (I) duration increased the magnitude of the outward tail currents also increased.

Pulse (I) command step to +50mV.

For a pulse (I) command step to +50mV, as the pulse (I) duration was increased the tail currents became markedly more inward for a given pulse (II) command potential (Fig. 3.2422 2A).

The corresponding I-V relationships (Figs. 3.2422 2Bi and ii) showed that increasing the pulse (I) magnitude from 10ms to 50ms resulted in the outward tail currents becoming more inward, thereby causing a positive shift in the reversal potential. Furthermore, a 50ms pulse (I) duration produced an inward current between -120mV and -56mV which peaked between -80 and -70mV.

With current measurements taken at 5ms and 10ms the trends were similar. Once again, however, the shift in reversal potential was more pronounced earlier on in the tail current i.e. for measurements taken at 5ms.

A plot of the reversal potential versus the pulse (I) duration shows a linear increase in reversal potential (Fig. 3.2422 3) as a function of pulse (I) duration. A straight line through the points was drawn using the Harvard Graphics (Microsoft) line trend function. The assumption is that for a 40ms increase in pulse duration between 10 and 50ms the increase in reversal potential was linear. If this

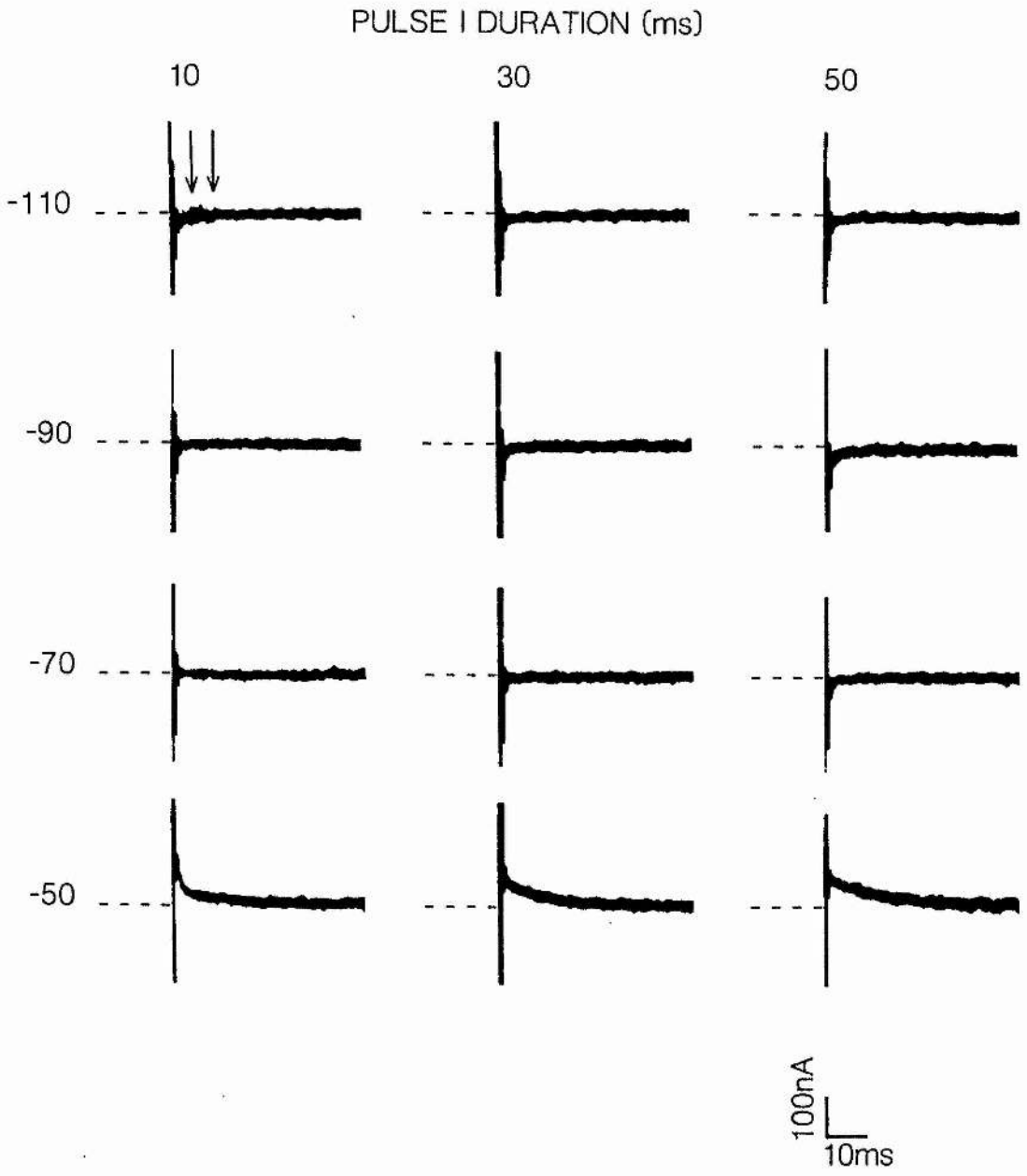


Fig. 3.2422 1A

The effect of pulse (I) duration on the tail current response: pulse (I) command step to 0mV.

Selected tail current responses taken at pulse (II) command potentials of -110, -90, -70 and -50mV.

Increasing the pulse duration had little effect on the tail current response for a pulse (I) potential of 0mV.

Holding potential: -70mV.

Arrows denote the position of current measurements: 5 and 10ms.

Fig. 3.2422 1B

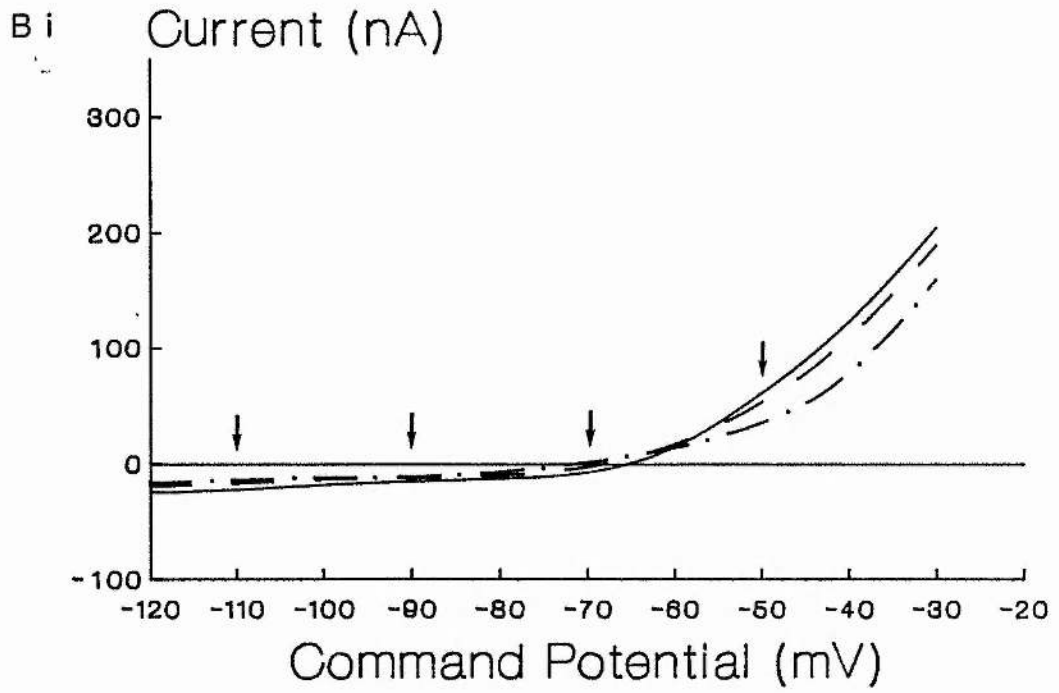
The effect of pulse (I) duration on the tail current I-V relationship: pulse (I) command step to 0mV.

Curves represent pulse (I) durations of 10ms (dot-dashed line), 30ms (dashed line) and 50ms (solid line).

i. Current measurements taken at 5ms. Tail currents for 10, 30 and 50ms reversed at -70, -68 and -65mV respectively.

ii. Current measurements taken at 10ms. Tail currents for 10, 30 and 50 reversed at -71, -71 and -65mV respectively.

As the pulse (I) duration increased the outward tail currents were also increased accompanied by a positive shift in reversal potentials.


B i
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)

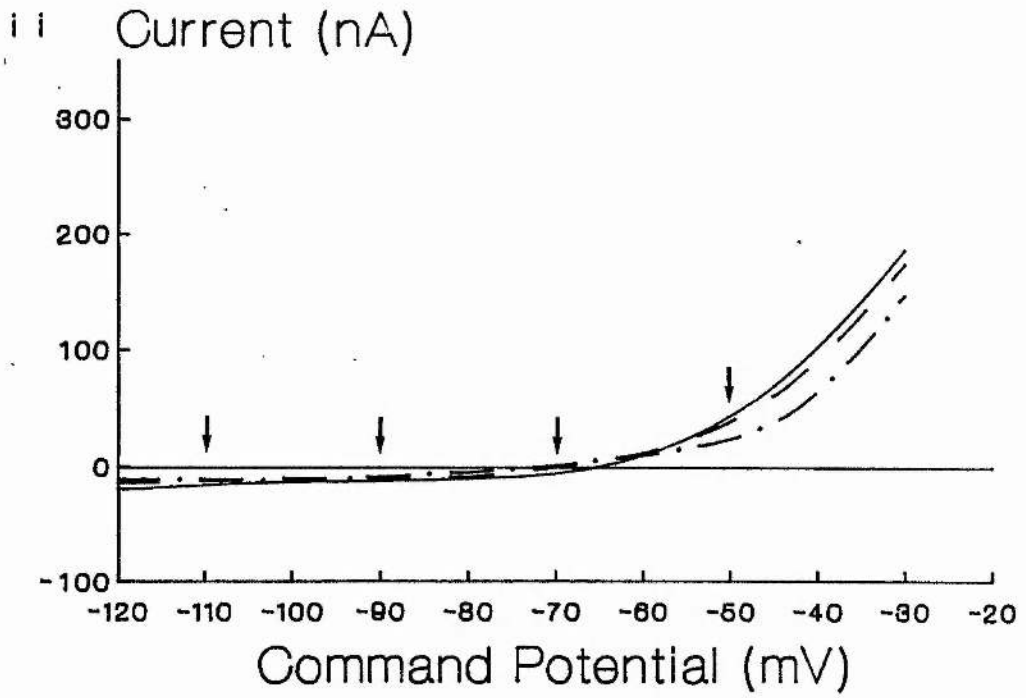

i i
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)

Fig. 3.2422 2A

The effect of pulse (I) duration on the tail current response: pulse (I) command step to +50mV.

Selected tail current responses taken at pulse (II) command potentials of -110, -90, -70 amd -50mV.

Increasing the pulse (I) duration caused the tail currents to become more inward for a given command potential.

Holding potential: -70mV.

Arrows denote the position of current measurements: 5 and 10ms.

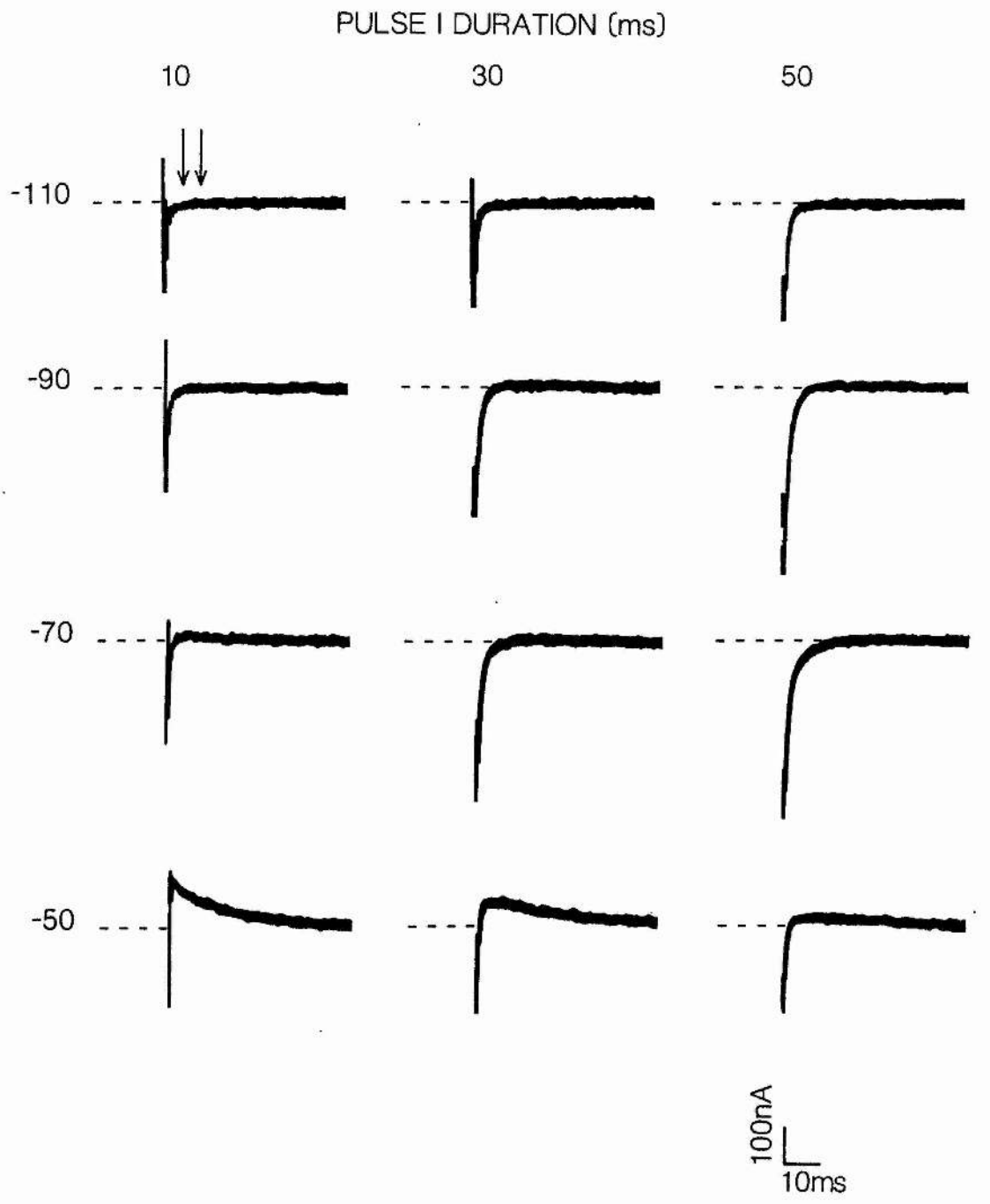

PULSE I DURATION (ms)
10
30
50
-110
-90
-70
-50
100nA
10ms

Fig. 3.2422 2B

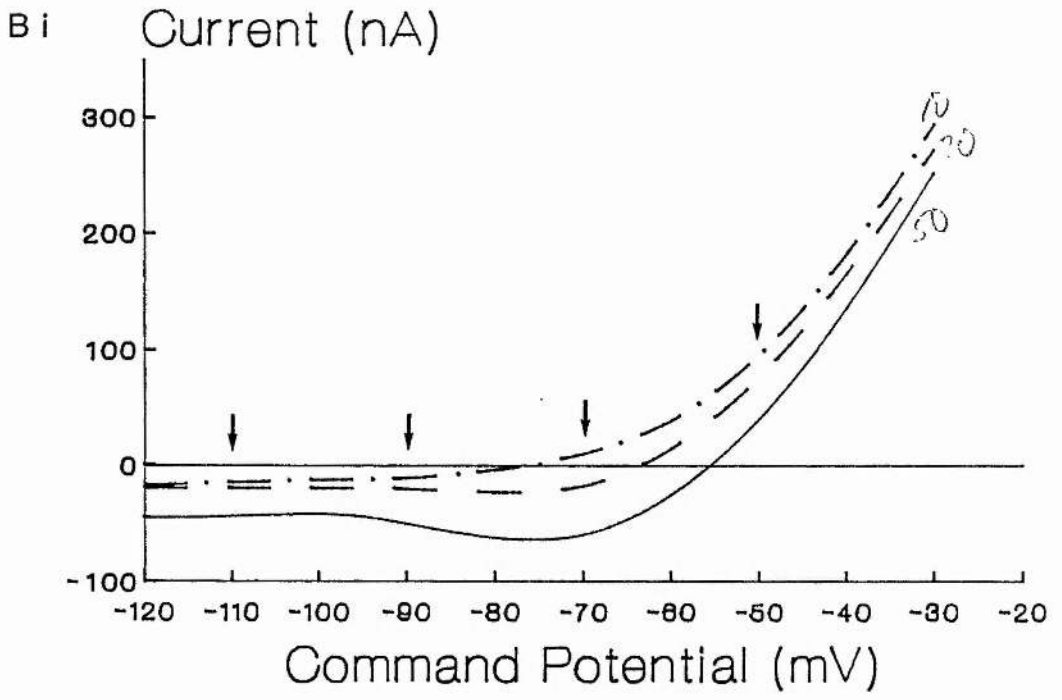
The effect of pulse duration on the tail current I-V relationship: pulse (I) command step to +50mV.

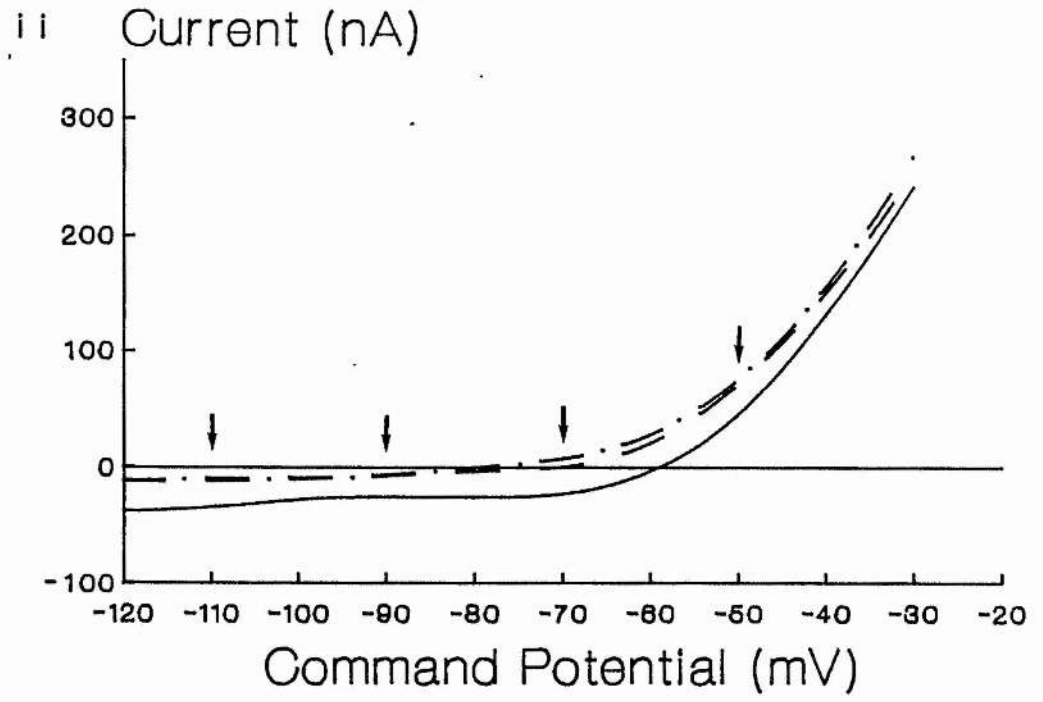
Curves represent pulse (I) durations of 10ms (dot-dashed line), 30ms (dashed line) and 50ms (solid line).

As the pulse (I) duration was increased the tail currents were reduced and the reversal potentials were shifted toward more positive potentials. This trend was more exaggerated with current measurements taken at 5ms.

i. Current measurements taken at 5ms. The reversal potential for pulse (I) durations of 10, 30, and 50ms were -76, -63 and -56mV respectively. Furthermore, pulse (I) duration of 50ms evoked a greater inward tail current between pulse (II) command potentials of -120 and -56mV which peaked between -80 and -90mV.

ii. Current measurements taken at 10ms. The reversal potential for pulse (I) durations of 10, 30 and 50ms were -80, -71 and -59mV respectively.


Bi
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)


ii
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)

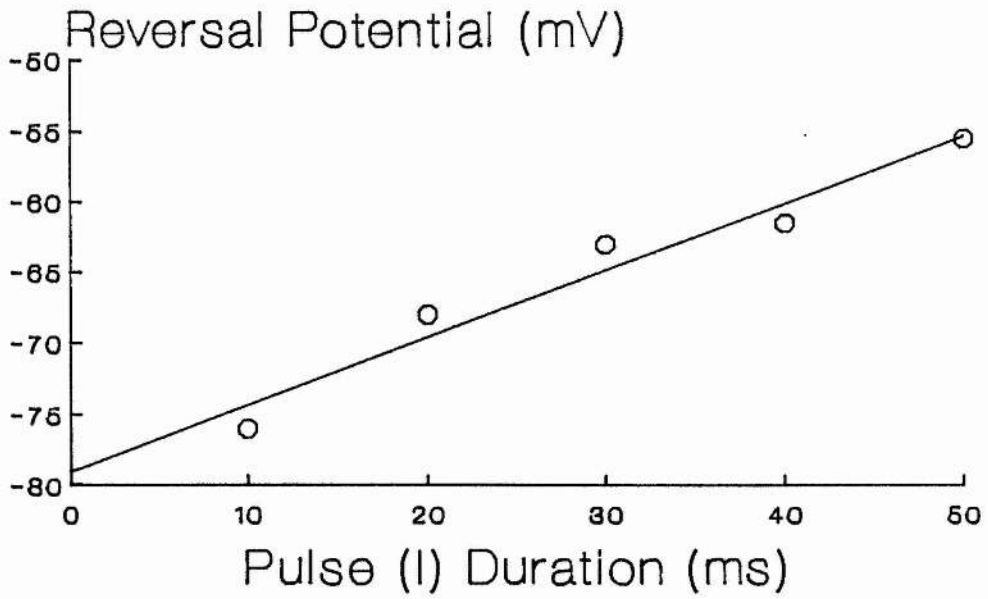


Fig. 3.2422 3

The relationship between reversal potential and pulse (I) duration.

Pulse (I) command step = +50mV. Reversal potential data taken from Fig. 3.2322 2B.

The linear relationship for a 40ms increase in pulse (I) duration between 10 and 50ms represented a two-fold change in extracellular cation or anion concentration.

is true then from the Nernst equation a 21.5mV change in reversal potential from -74 to -55.5mV represents an approximate two-fold cation accumulation or anion depletion in the extracellular space. From these experiments it was not clear whether the two-fold change in external ion concentration would occur for pulse (I) durations greater than 50ms. This does not exclude the possibility that the values shown here form part of a shallow curve.

Pulse (I) command step to +100mV.

For a pulse (I) command step to 100mV increasing the pulse (I) duration produced a similar effect as for pulse (I) 50mV but the inward trend in tail currents was not as pronounced (compare Fig. 3.2422 4A with Fig. 3.2422 2A). There was also a smaller positive shift in reversal potential (Figs. 3.2422 4Bi and ii).

Tables 3.2422 1A and B provide a summary of the provisional results on the effect of pulse (I) duration on the tail current reversal potentials. Further work is necessary to provide a statisical analysis of the data.

Fig. 3.2422 4A

The effect of pulse (I) duration on the tail current response: pulse (I) command step to +100mV.

Selected tail current responses taken from pulse (II) command potentials of -110, -90, -70 and -50mV

Increasing the pulse (I) duration caused the tail currents to become more inward for a given command potential. The trend was similar to that observed for a pulse (I) command step to 50mV (Fig. 3.2322 2A).

Holding potential: -70mV.

Arrows denote the position of current measurements: 5 and 10ms.

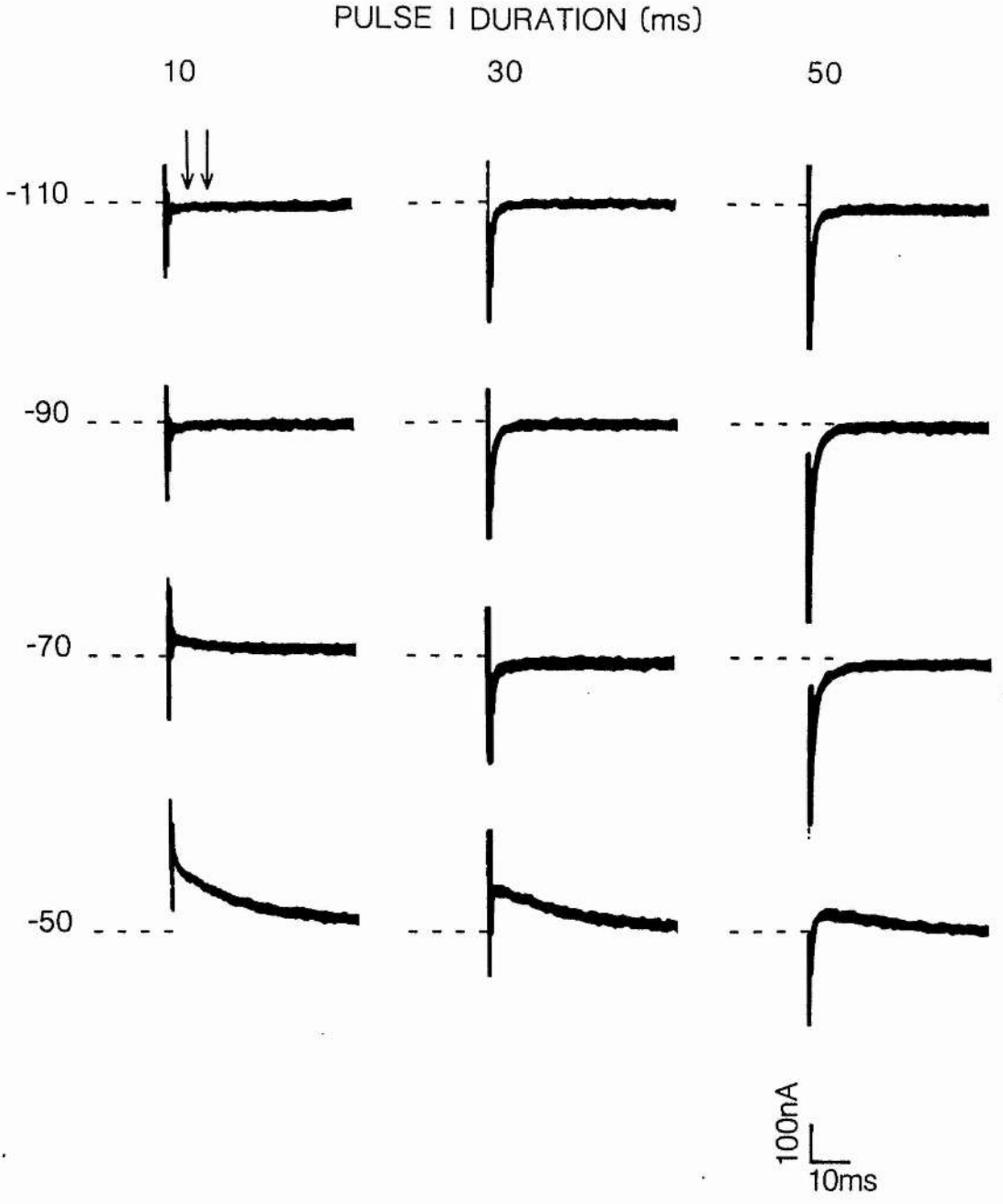

PULSE I DURATION (ms)
10
30
50
-110
-90
-70
-50
100nA
10ms

Fig. 3.2422 4B

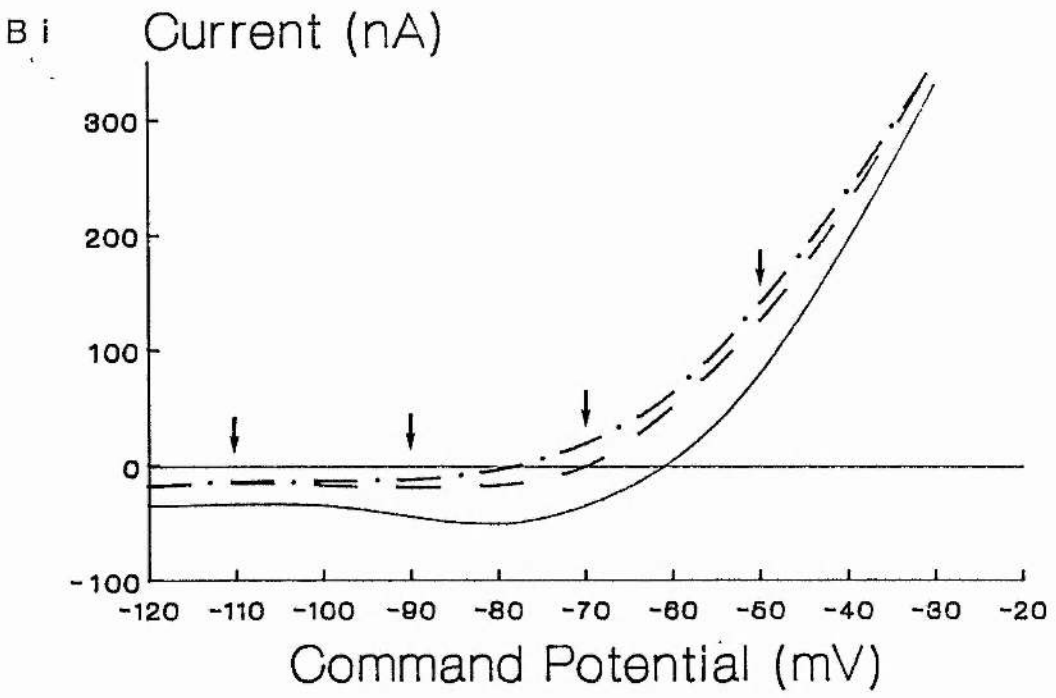
The effect of pulse (I) duration on the tail current I-V relationship: pulse (I) command step to +100mV.

Curves represent pulse (I) durations of 10 (dot-dashed line), 30 (dashed line) and 50ms (solid line).

As the pulse (I) duration was increased the tail currents were reduced and the reversal potentials were shifted toward more positive potentials. This trend was more exaggerated with current measurements taken at 5ms.

i. Current measurements taken at 5ms. The reversal potentials for pulse (I) durations of 10, 30 and 50ms were -79, -70 and -61mV respectively. Furthermore, a pulse (I) duration of 50ms evoked a greater tail current between pulse (II) command potentials of -120 and -60mV.

ii. Current measurements taken at 10ms. The reversal potentials for pulse (I) durations of 10, 30 and 50ms were -77, -77 and -69mV respectively.


B i
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)

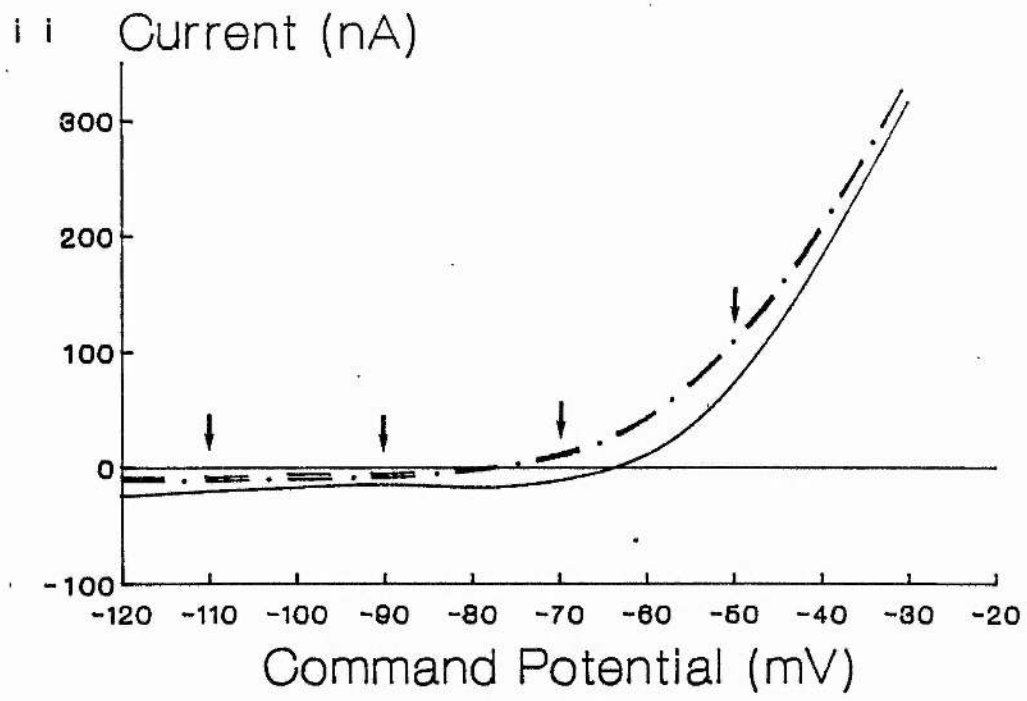

i i
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)

Table 3.2422 1

Summary of the effect of pulse (I) duration on the reversal potential for tail current measurements taken at A.) 5ms and B.) 10ms.

	Pulse (I) Magnitude (mV)	Pulse (I) Duration (ms)		
		10	30	50
A.	0	-70	-68	-65
	50	-76	-63	-56
	100	-79	-70	-61
B.	0	-71	-71	-65
	50	-80	-71	-59
	100	-77	-77	-65

3.2423 Cadmium.

External application of cadmium (1mM) dramatically reduced the net outward current evoked by a pulse (I) command step to +50mV (not shown). The accompanying outward tail currents were also reduced (Fig. 3.2423 1A). Washing out Cd^{2+} resulted in partial recovery of the current response during pulse (I). In this experiment, however, a steady inward tail current now occurred at more negative potentials during pulse (II). There was partial recovery of outward tail currents at more positive potentials. The tail current response is sensitive to external saline disturbance during washing. In some, but not all, experiments involving saline perfusion there was an increase in the magnitude of inward and particularly outward tail currents suggesting that the neuronal membrane had been damaged in some way.

The corresponding I-V relationship for measurements taken at 5ms and 10ms (Figs. 3.2423 1Bi and ii) show the marked reduction in the outward tail current after Cd^{2+} treatment. The remaining current showed an almost ohmic relationship with command potential. Washout with normal saline caused an increase in inward currents at more negative potentials and a partial recovery of the outward tail currents. The reversal potential was not affected by external Cd^{2+}.

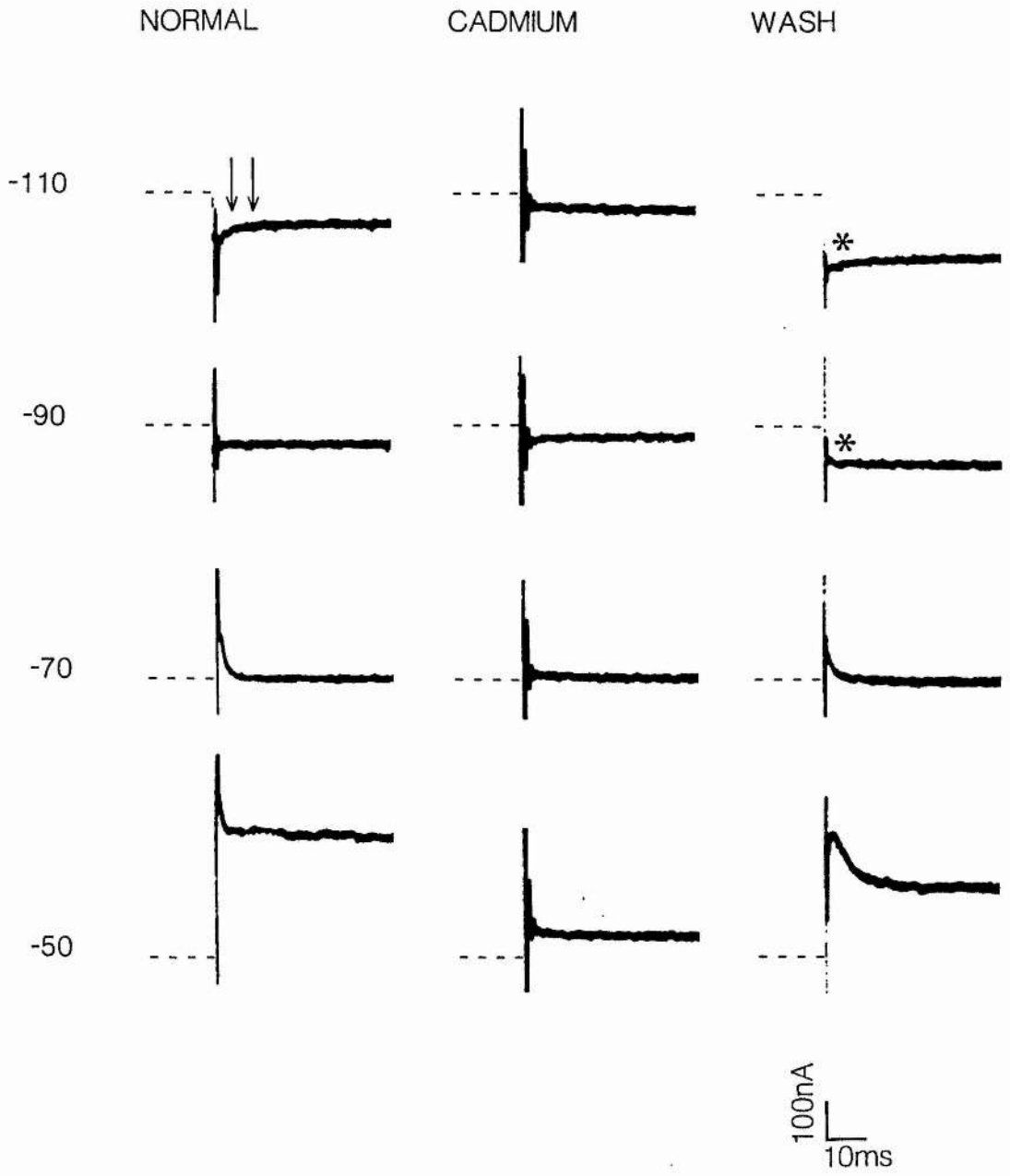


Fig. 3.2423 1A

The effect of cadmium on the tail current response.

Selected tail current responses taken at pulse (II) potentials of -110, -90, -70 and -50mV. A standard 20ms pulse (I) command step to +50mV.

Cadmium (1mM) caused an overall reduction in tail currents. The outward tail current response was partially restored after 15mins washout with normal saline, though there was an increase in inward currents (asterisk) with respect to the normal run.

Holding potential: -70mV.

Arrows denote the position of current measurements: 5ms and 10ms.

Fig. 3.2423 1B

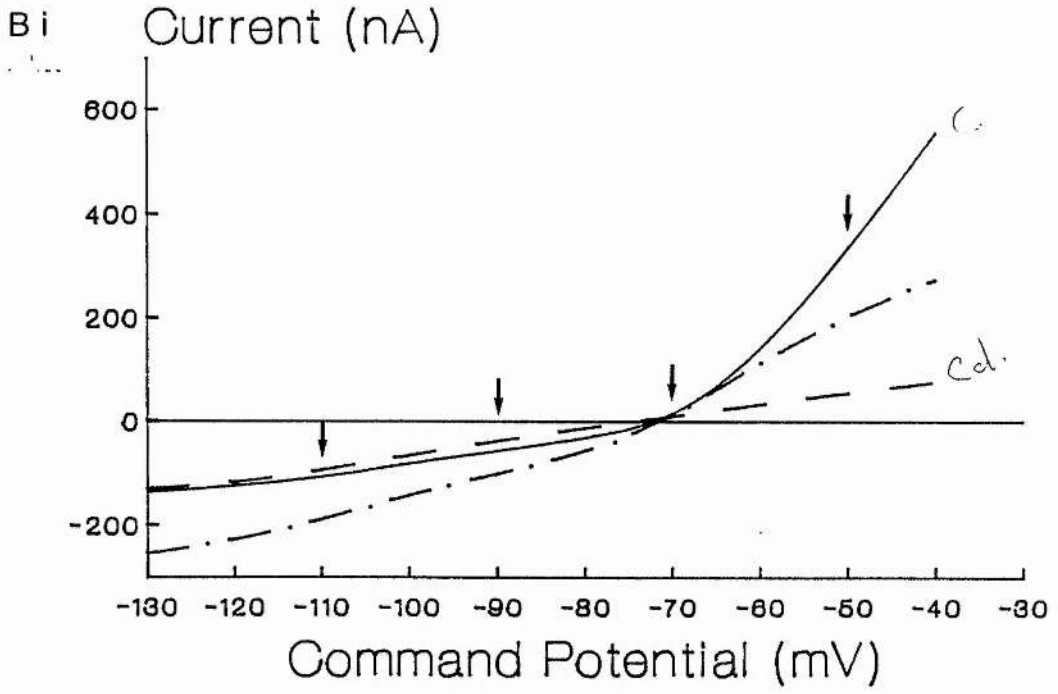
The effect of cadmium on the tail current I-V relationship.

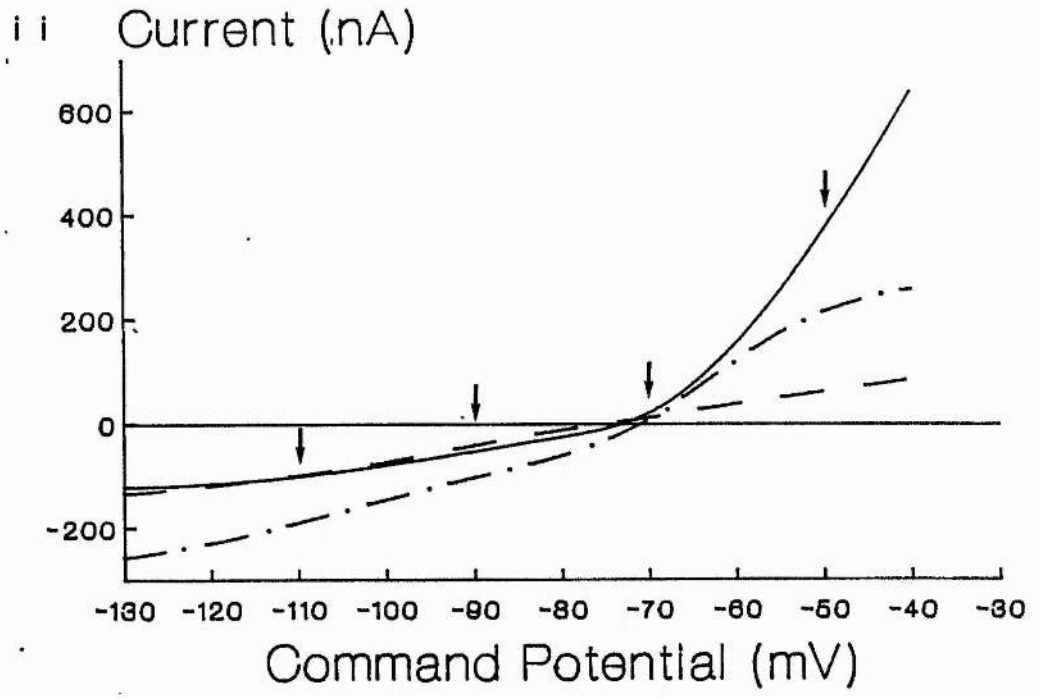
Curves represent normal run (solid line), 10mins. Cd^{2+} (1mM) (dashed line) and finally, after 15mins. wash with normal saline (dot-dashed line). Pulse (I) command step to +50mV and 20ms duration.

Cadmium ions caused a dramatic reduction in outward tail currents causing the I-V relationship to become almost ohmic. Washout did partially restore the outward tail currents but inward tail currents were now increased.

i. Current measurements taken at 5ms. The reversal potential of -72mV was relatively unaffected by cadmium treatment or washout.

ii. Current measurements taken at 10ms. The reversal potential was shifted -2mV by cadmium treatment and +1mV by washout with respect to the normal run at -73mV.


Bi
Current (nA)
600
400
200
0
-200
-130 -120 -110 -100 -90 -80 -70 -60 -50 -40 -30
Command Potential (mV)


ii
Current (nA)
600
400
200
0
-200
-130 -120 -110 -100 -90 -80 -70 -60 -50 -40 -30
Command Potential (mV)

3.2424 External ion concentration manipulation

Using a double command pulse regime tail current reversal potential lay between -60 and -80mV depending on the pulse (I) magnitude and duration as shown above. The reversal potential alone was not sufficient to identify the ionic basis of the outward current since potassium and chloride ions are likely to have an equilibrium potential within this command potential range. The outward current during depolarisation could, therfore, be due to either potassium ions leaving or chloride ions entering the cell body. Altering the external ion concentration should, in theory, produce a shift in reversal potential predicted by the Nernst equation for a current dependent on that particular ion species.

3.24241 High external K^+ concentration.

According to the Nernst equation a five-fold increase in external K^+ (from 3.1 to 15mM) ought to produce a +40mV shift in reversal potential for a potassium-dependent conductance. Tail currents from a standard pulse (I) duration of 20ms and command step to +50mV, however, showed a small variable change in reversal potential. From four experiments the maximum shift observed was +17mV (Fig. 3.24241 1).

3.24242 Low external Cl^- concentration.

According to the Nernst equation a 20% reduction in external Cl^- i.e. from 235mM to 47mM ought to produce a +40mV shift in reversal potential for a chloride-dependent conductance. The overall size and shape of tail currents from a pulse (I) duration of 20ms and command step to +50mV were not appreciably affected, although the initial fast inward tail current was increased between command potentials of -110 and -90mV (arrowheads Fig. 3.24242 1A).

The corresponding I-V relationship shows that there was no significant shift in reversal potential during low chloride saline

treatment (Figs. 3.24242 1Bi and ii). The increase in inward and outward tail current magnitude after low chloride saline perfusion and wash may have been due to impalement disurbance caused by the superfusing saline.

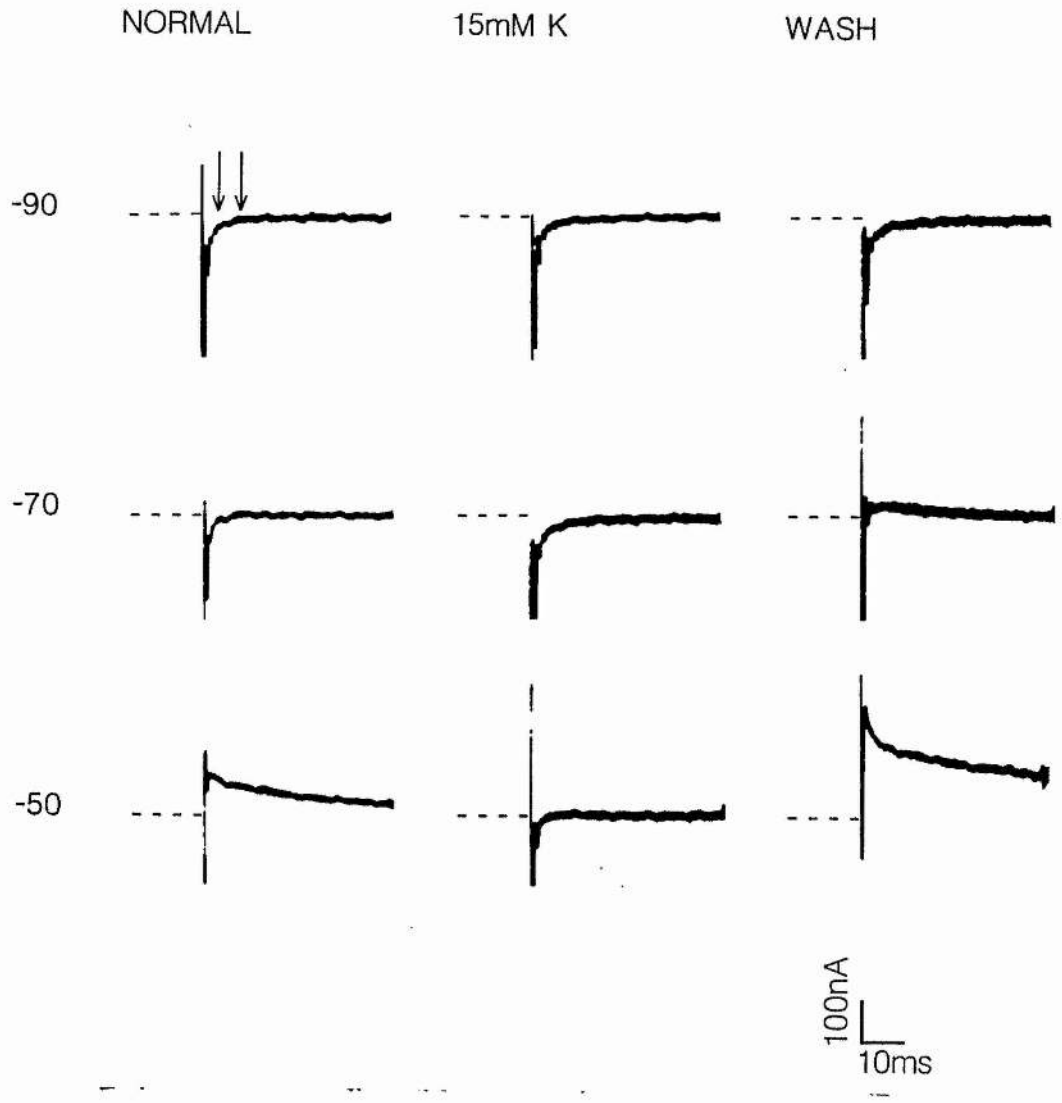


Fig. 3.24241 1A

The effect of a five-fold increase in external potassium ion concentration on the tail current response.

Selected tail current responses from pulse (II) potentials of -90, -70 and -50mV. A standard 20ms pulse (I) command step to +50mV.

A five-fold increse in external potassium ion concentration from 3.1 to 15mM caused the tail currents to be less outward. In this experiment, replacement with normal saline then caused an increase in outward tail currents in excess of the normal response.

Holding potential: -70mV.

Fig. 3.24241 1B

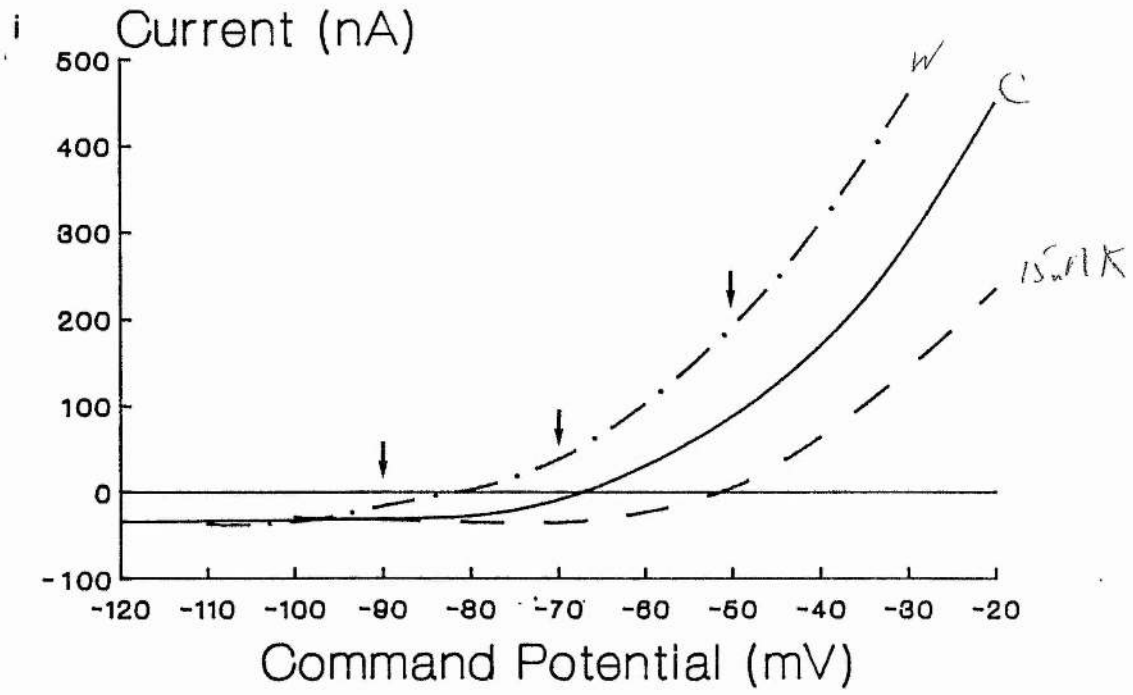
The effect of a five-fold increase in external potassium ion concentration on the tail current I-V relationship.

Curves represent normal run (solid line) after 20mins. 15mM K^+ saline and, finally, replacement with normal saline (dot-dashed line).

i.. Current measurements taken at 5ms: high potassium saline caused a +16mV shift in the reversal potential from -67 to -51mM. After replacement with normal saline the reversal potential then shifted to -82mV.

ii.. Current measurements taken at 10ms: high potassium saline caused a +18mV shift in the reversal potential from -72 to -54mM. After replacement with normal saline the reversal potential then shifted to -79mV.

Note that the large negative shift in reversal potential was not a feature of high potassium saline treatment and may be due to impalement disturbance caused by superfusing saline.


i
Current (nA)
500
400
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)
W
C
ISwIK

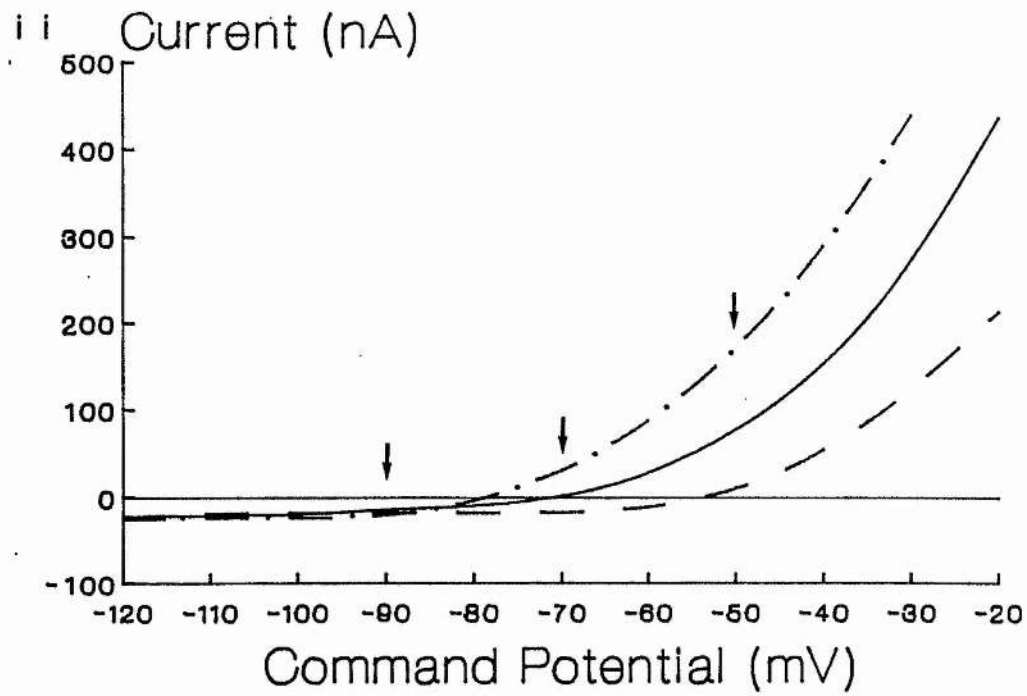

i i
Current (nA)
500
400
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)

Fig. 3.24242 1A

The effect of a five-fold reduction in external chloride ion concentration on the tail current response.

Selected tail currents taken from pulse (II) potentials of -110, -90, -70 and -50mV. A standard 20ms pulse (I) command step to +50mV.

Reducing the external chloride ion concentration from 235mM to 47mM had very little effect on the tail currents. In this experiment the initial fast inward tail current was increased between pulse (II) command potentials of -110 and -90mV (arrowheads).

Holding potential: -70mV.

Arrows denote the position of current measurements: 5 and 10ms.

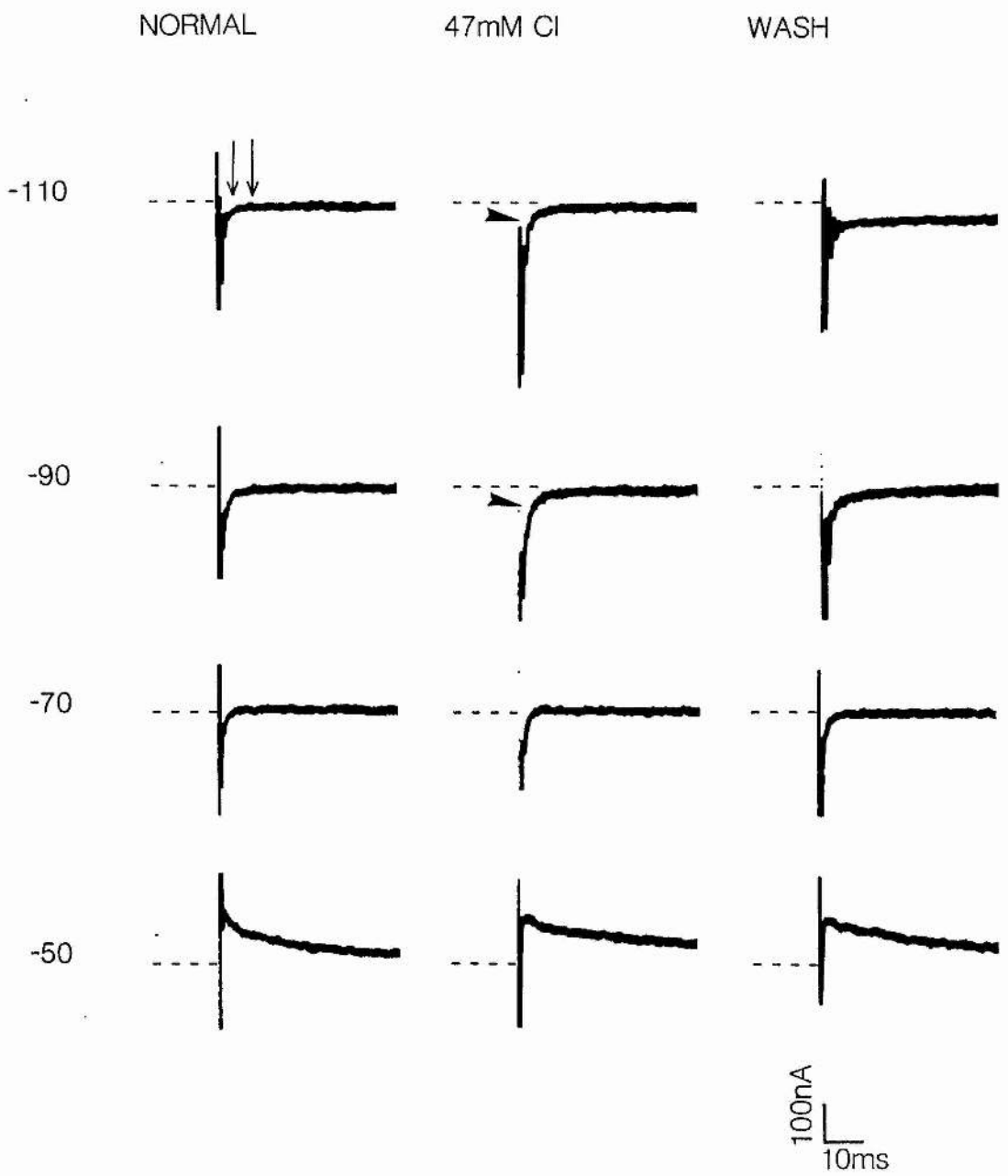
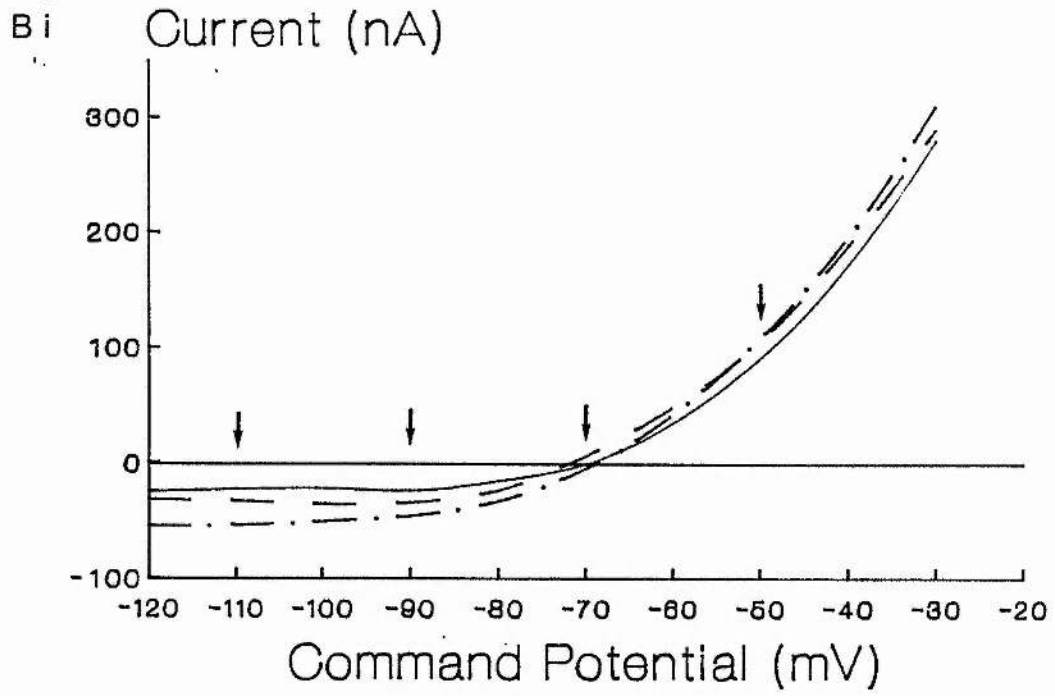

NORMAL
47mM Cl
WASH
-110
-90
-70
-50
100nA
10ms

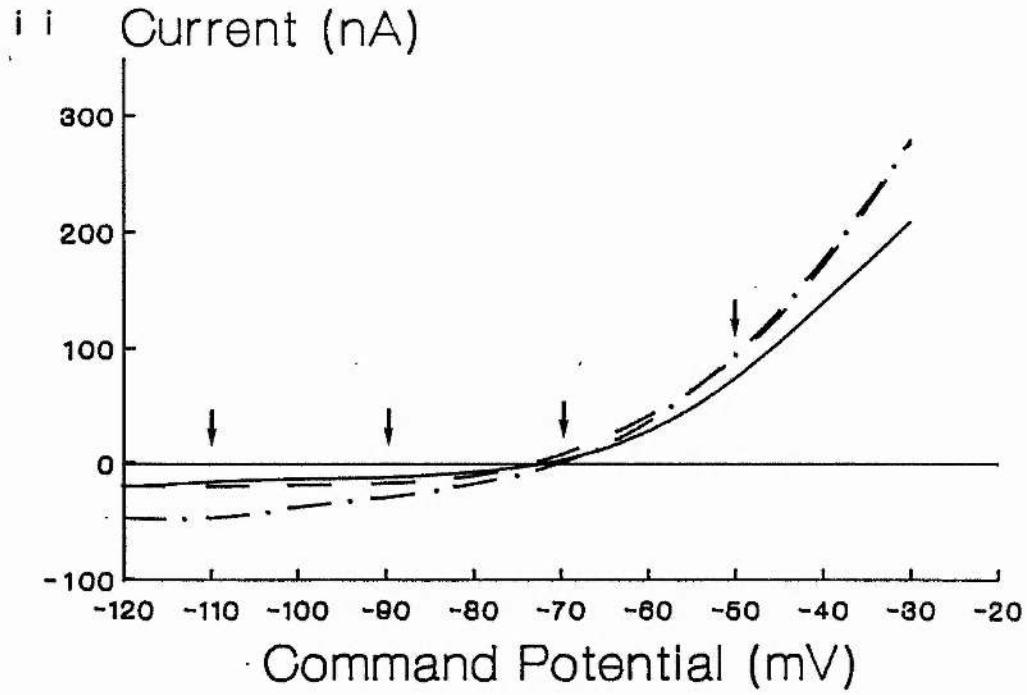
Fig. 3.24242 2A

The effect of a five-fold reduction in external chloride ion concentration on the tail current I-V relationship.

Curves represent normal run (solid line) followed by 47mM chloride saline (dashed line) and, finally, replacement with normal saline (dot-dashed line).

The reversal potential for tail current measurements taken at i. 5ms was -70mV and at ii. 10ms was -72mV. Neither low chloride saline or replacement with normal saline caused a significant change in reversal potential.


Bi
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)


ii
Current (nA)
300
200
100
0
-100
-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20
Command Potential (mV)

3.3 CURRENT-CLAMP.

When depolarised under voltage-clamp cell 3 produces a strong outward current underlying the characteristic inexcitability of the cell body under current-clamp. There are, however, a number of procedures which are known to enhance the excitability of other preparations (see section 1.3) including insect neurones Pitman, 1975; 1979; 1988; Goodman and Heitler, 1979). Some of these acute and chronic procedures are described below.

3.31 Normal animal.

Under normal conditions injecting depolarising current into the cell body of cell 3 did not produce an action potential. Instead a series of dampened oscillations were evoked (Fig. 3.31 1). The amplitude of these membrane voltage oscillations depended on the magnitude of applied current. Under these conditions the cell body was unable to spike even with large depolarisations.

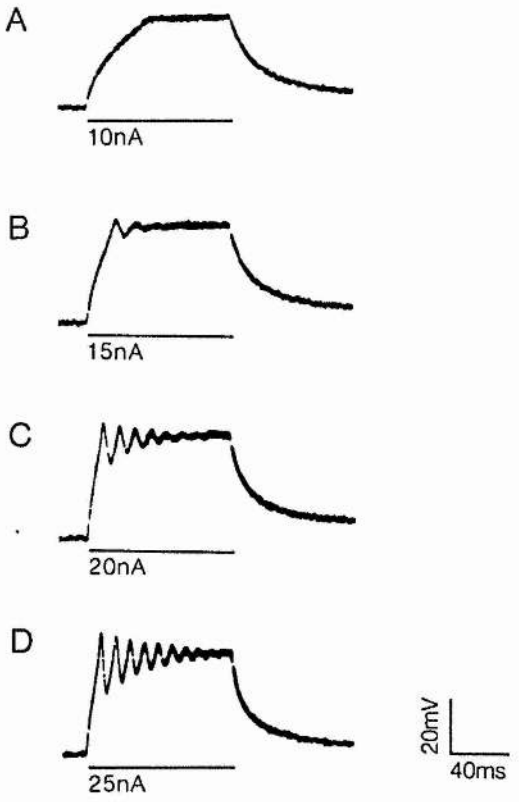


Fig. 3.31 1

Normal voltage oscillation of the soma membrane in response to intracellular depolarisation.

A - D. The magnitude of the membrane oscillations increased with applied current.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -61mV.

3.32 ACUTE CHANGES IN ELECTRICAL PROPERTIES.

3.321 TEA^+ treatment.

The inexcitable oscillatory state was converted into an excitable state after acute external application of TEA^+. After 5-10mins. treatment with TEA^+ (50mM), a small depolarising current produced a broad all-or-nothing action potential (Fig. 3.321 1). The ionic basis of this TEA^+-induced action potential was not investigated. A previous study on the cell body of another cockroach motoneurone, however, demonstrated calcium to be carrying the inward current (Pitman, 1979).

3.322 Aminopyridines

The aminopyridines have been reported to increase excitability in locust neurones (Gooman and Heitler, 1979). External application of 5mM 4-AP and 3,4-DAP induced a progressive slow depolarisation which was accompanied by spontaneous epsps (Fig. 3.322 1). After several minutes drug treatment the resting potential further depolarised and the membrane oscillatory response was abolished. In this experiment, the membrane potential underwent a +11mV depolarisation from -68mV to -57mV. There was a large reduction in the voltage response to the same magnitude of applied current (+20nA) that once caused membrane oscillation, indicating a corresponding large fall in input resistance of the cell body. Washout with normal saline led to partial recovery of the oscillatory response during strong depolarisations (+75nA).

In view of the enhanced excitability that aminopyridines cause in locust neurones (Goodman and Heitler, 1979) the loss of the oscillatory response and concomitant depolarisaton were unexpected effects. To test whether the excitability of the cell was still capable of enhancement TEA^+ was externally applied to the preparation. In this experiment subsequent application of TEA^+

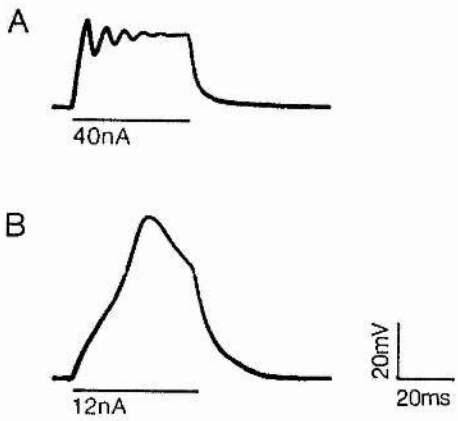


Fig. 3.321 1

The effect of externally applied TEA^+ on the membrane response during intracellular depolarisation.

A. Intracellular current injection (40nA) produced a series of membrane voltage oscillations.

B. Within 5mins., externally applied TEA^+ (50mM) now enabled a relatively small depolarising pulse (12nA) to evoke a broad action potential.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -61mV.

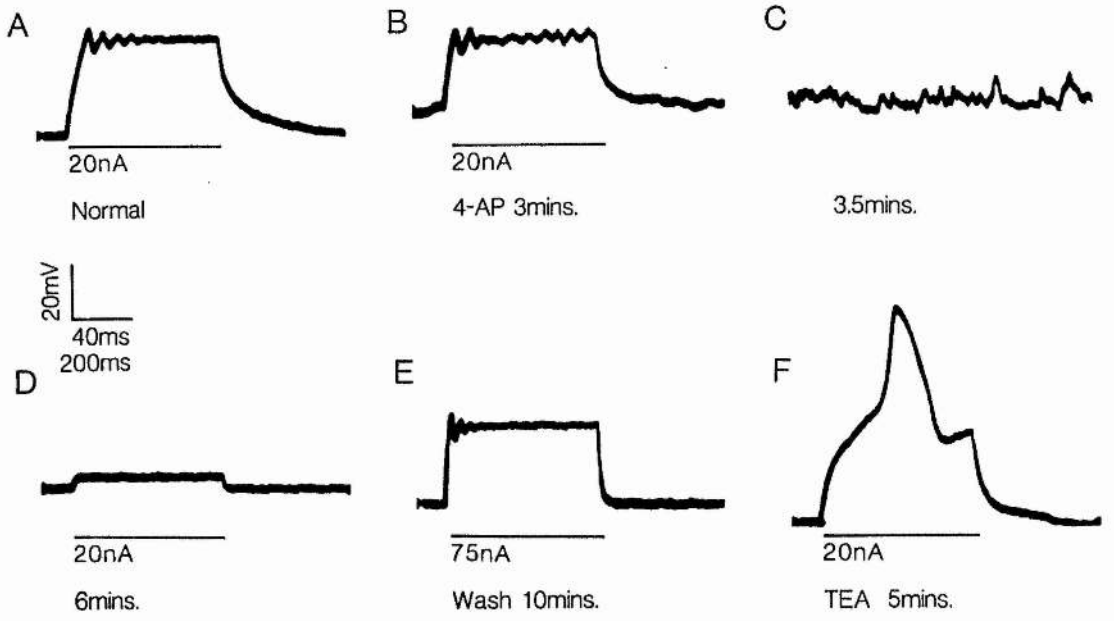


Fig. 3.322 1

The effect of externally applied 4-AP on the membrane response during intracellular depolarisation.

A. Normal membrane rsponse to +20nA intracellular depolarisation. Resting potential = -68mV.

B. Within 3mins., 4-AP (5mM) caused the membrane response to decline and become irregular. Resting potential = no data.

C. The membrane potential now showed spontaneous epsps of up to 10 mV amplitude. Scale bar 200ms. Resting potential = -58mV.

D. With continued drug treatment the membrane oscillation disappeared corresponding to a massive fall in input resistance. Resting potential = -57mV.

E. After 10mins. wash with normal saline a strong depolarising current (+75nA) was able to evoke a small membrane oscillation. Resting potential = -62mV

F. Within 5mins. externally applied TEA^+ (25mM) was able to block ion channels and cause the appearance of a broad action potential in response to a relatively small depolarising current (20nA). Resting potential = -69mV.

Bar underneath represents the current pulse duration. Current intensities shown underneath.

produced a characteristic broad action potential of approximately 35ms. The full duration of the TEA^+-induced action potential could be observed with a 100ms current pulse.

3.323 Intracellular citrate.

Micropipettes were filled with tri-potassium citrate (1M) and hyperpolarising pulses (duration one second) applied at a frequency of 0.5Hz. Intracellular ionophoretic injection of citrate ions by this method produced all-or-nothing action potentials within a few minutes. The action potential amplitude varied between 44 to 75mV and between 5 to 8ms duration (Fig. 3.323 1) these action potentials were thus faster than the TEA^+-induced action potential.

Intracellular citrate ions are thought to chelate a proportion of cytosolic calcium ions. Reducing the intracellular calcium concentration near the membrane will have two important consequences:

i) The driving force on external Ca^+ will be increased.

ii) The activation of a calcium-dependent outward current will be reduced.

These two effects, in conjunction, could unmask a regenerative inward current. The ionic basis of the citrate-induced action potential was investigated.

Na-free saline either had no appreciable effect or, as in this experiment, caused a small increase in the action potential (Fig. 3.323 2). A 4-fold increase (9mM to 36mM) in external calcium ion concentration caused only a small increase in the citrate-induced action potential (Fig 3.323 3). Blocking the calcium influx did, however, reversibly reduce or abolish the citrate-induced action potential. Externally applied verapamil (10uM) reduced the regenerative component of the action potential to a small graded spike, whereas Mn^{2+} (40mM) completely abolished the regenerative

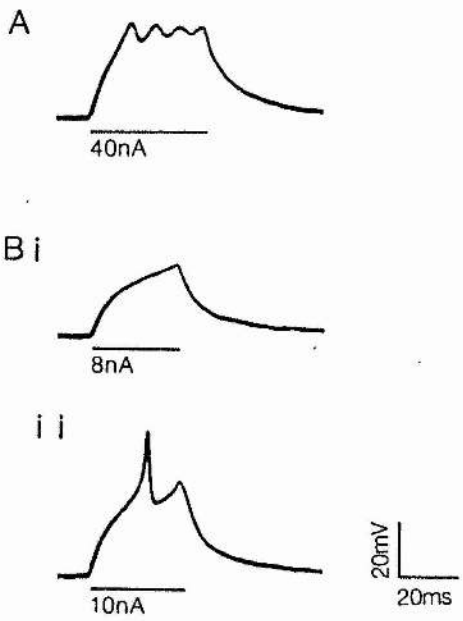


Fig.3.323 1

The effect of ionophoretic injection of citrate ions into the soma of cell 3.

A. Normal membrane oscillation in response to depolarising current (+40nA).

B. Within 5mins., intracellular citrate now caused the appearance of an all-or-nothing action potential in response to intracellular depolarisation.

i.) A sub-threshold 8nA current injection causes a passive membrane response.

ii.) A 10nA current injection is able to evoke an action potential.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -59mV.

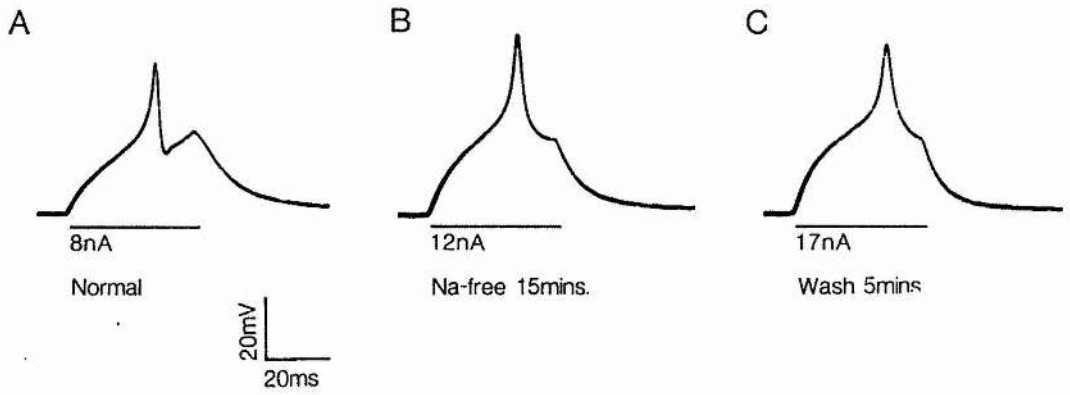


Fig. 3.323 2

The effect of Na-free saline on the citrate-induced action potential.

A. Normal citrate-induced action potential.

B. After 15mins. Na-free saline had very little effect on the action potential. In this experiment there is a slight enhancement of the action potential.

C. After 5mins. wash with normal saline.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -59mV.

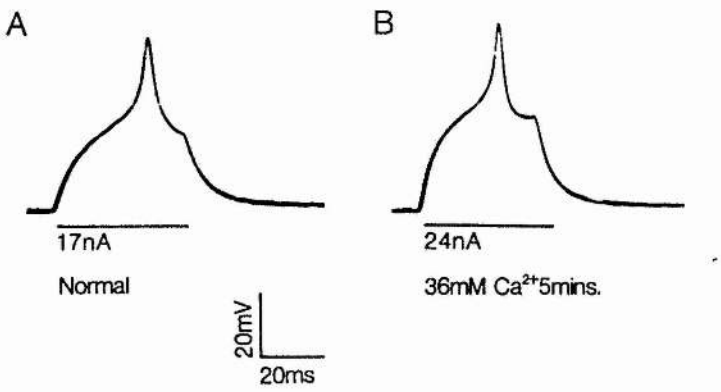


Fig. 3.323 3

The effect of high calcium saline on the citrate-induced action potential.

A. Normal citrate-induced action potential.

B. High calcium saline (36mM) caused a slight increase in the action potential.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -64mV.

component (Figs. 3.323 4 and 5). A graded spike was broad, showed no upward inflection on the rising phase characteristic of a regenerative component and increased in amplitude with applied current. These findings provide more evidence for calcium as the major ion carrying the inward current under these conditions.

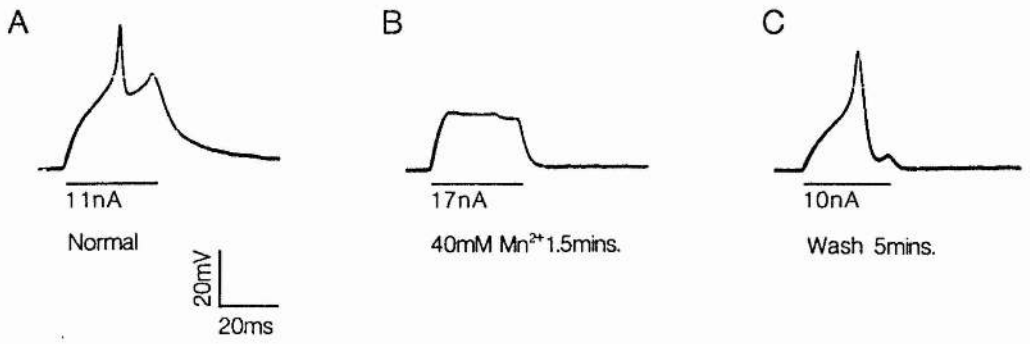


Fig. 3.323 4

The effect of Mn^{2+} saline on the citrate-induced action potential.

A. Normal citrate-induced action potential.

B. After 1.5mins. Mn^{2+} (40mM) treatment the action potential was completely abolished.

C. Restoration of the action potential after 5 ins. wash with normal saline.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -59mV.

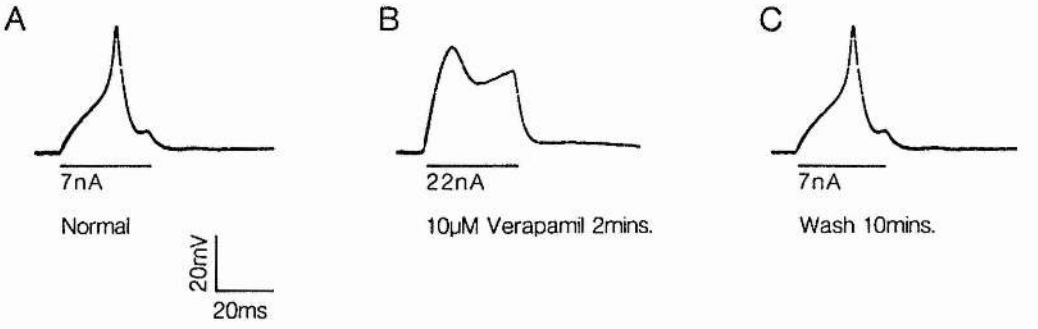


Fig. 3.323 5

The effect of verapamil on the citrate-induced action potential.

A. Normal citrate-induced action potential.

B. After 2mins. verapamil (10μM) treatment reduced the regenerative component to a graded spike, despite increasing the applied current.

C. Restoration of the action potential after 10mins. wash with normal saline.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -59mV.

3.33 CHRONIC CHANGES IN EXCITABILITY.

3.331 CARBON DIOXIDE TREATMENT.

When animals had been anaesthetised in a 100% CO_2 atmosphere for 1-2 hours then removed allowed to recover, cell 3 was now enabled to sustain all-or-nothing action potentials when depolarising current was injected into the soma (Fig. 3.331 1). The action potentials were typically of +40 to +50mV amplitude and 3 to 5ms duration. The action potential rarely overshot zero mV. Much lower current magnitudes i.e. 6 to 12nA were required to produce these action potentials than were required to evoke oscillatory responses.

Although the capability for sustaining action potentials was usually present after 24 hours anoxia the earliest response was seen at 17 hours. Occasionally an early transition response was observed, presumably in preparations which had not yet developed complete excitability. The transition responses had a definite upwards inflection on the rising phase characteristic of a regenerative component (arrowhead Fig. 3.331 2). The magnitude of the transition response depended on the magnitude of applied current. These oscillations were large in comparison to the normal oscillatory response and more severely dampened.

The ionic basis of the carbon dioxide-induced action potential was investigated. Na-free saline caused a rapid reduction of the fast regenerative component and instead a broad graded spike persisted (Fig. 3.331 3). Tetrodotoxin (TTX) was originally reported to specifically block fast axonal Na-channels (Narahashi, Moore and Scott, 1964). Application of TTX (50nM) abolished the action potential (Fig. 3.331 4). The block was irreversible for wash periods up to 20mins. A 4-fold increase in external Ca^{2+} concentration had little effect on the action potential. In some preparations there was a slight reduction in the height of the

action potential in high Ca^{2+} solutions (Fig. 3.331 5). This may have been due to enhanced activation of the calcium-dependent outward current which would instigate premature repolarisation and truncation of the spike. Application of Mn^{2+} (40mM) reversibly reduced the action potential to a broad graded spike (Fig. 3.331 6). Often after treatment with Mn^{2+} the recovering action potential was broader than before, again probably due to blockade of a calcium-dependent outward current. Full recovery was achieved with prolonged washing.

From these results it appears that the inward current of the carbon dioxide-induced action potential was carried by sodium and calcium ions, though it is not clear if one ion makes more contribution than the other.

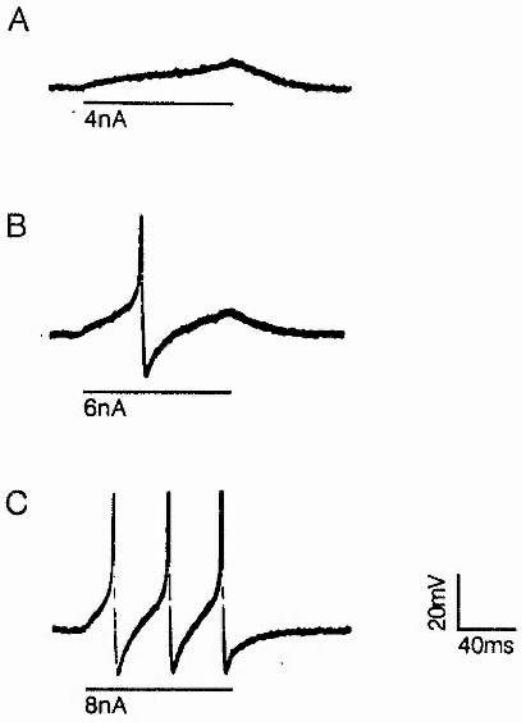


Fig. 3.331 1

The effect of whole-animal pre-treatment with carbon dioxide on the membrane response during intracellular depolarisation.

A - C. Gradually increasing the magnitude of applied current evoked an all-or-nothing action potential with a typical duration of 3 to 5ms.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -65mV.

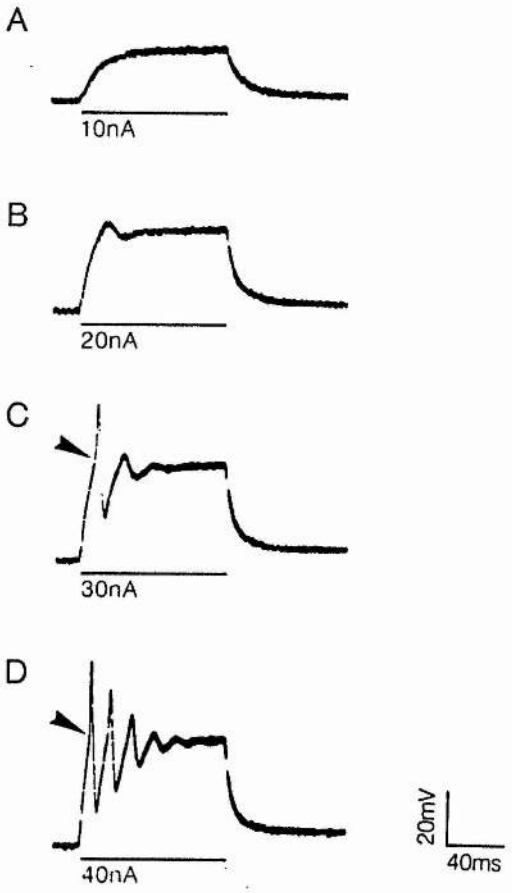


Fig. 3.331 2

A transition oscillatory response after whole-animal pre-treatment with carbon dioxide.

A - D. Gradually increasing the magnitude of applied current evoked a series of dampened membrane voltage oscillations. The magnitude of the response increased with applied current. The rising phase of the oscillations had an inflection reminiscent of a regenerative component (arrowhead).

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -65mV.

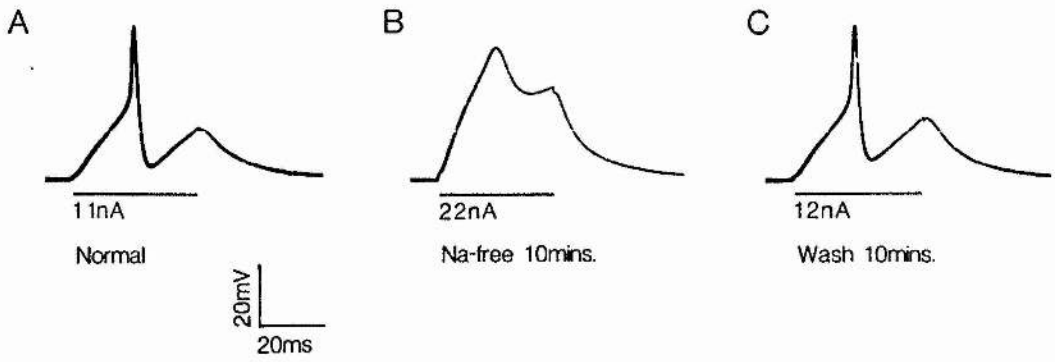


Fig. 3.331 3

The effect of Na-free saline on the carbon dioxide-induced action potential.

A. Normal carbon dioxide-induced action potential.

B. After 10mins., Na-free saline reduced the action potential to a broad graded spike.

C. Restoration of the action potential after 10mins. wash with normal saline.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -69mV.

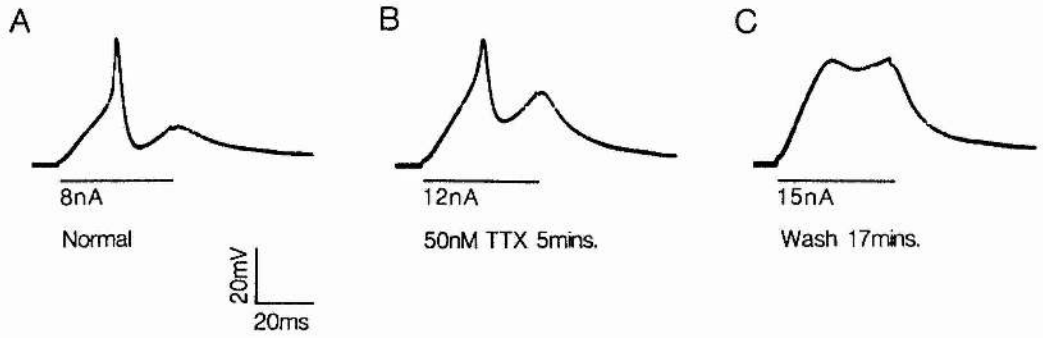


Fig. 3.331 4

The effect of TTX on the carbon dioxide-induced action potential.

A. Normal carbon dioxide-induced action potential.

B. After 5mins. TTX (50nM) treatment the amplitude of the action potential was beginning to decline and the regenerative phase was not so distinctive.

C. After 17mins. wash with normal saline the graded spike had further declined.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -72mV.

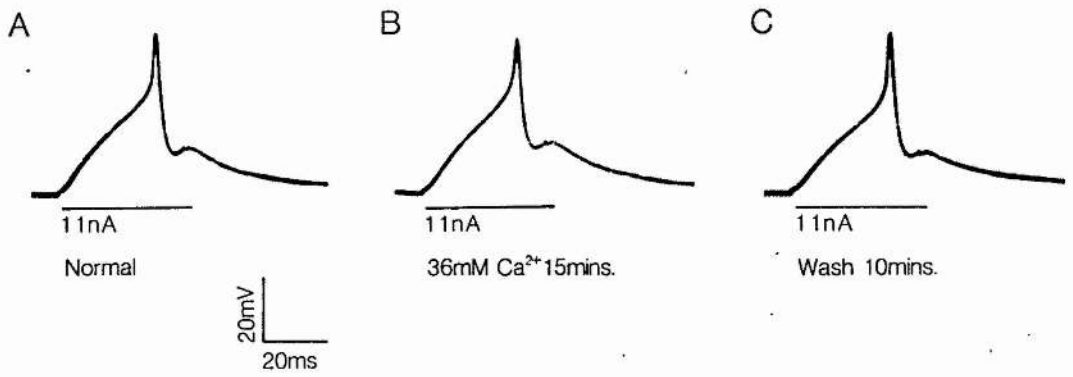


Fig. 3.331 5

The effect of high calcium saline on the carbon dioxide-induced action potential.

A. Normal carbon dioxide-induced action potential.

B. After 15mins., high calcium (36mM) saline had very little effect on the action potential. In this experiment there was a slight reduction in amplitude.

C. After 10mins. wash in normal saline.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -71mV.

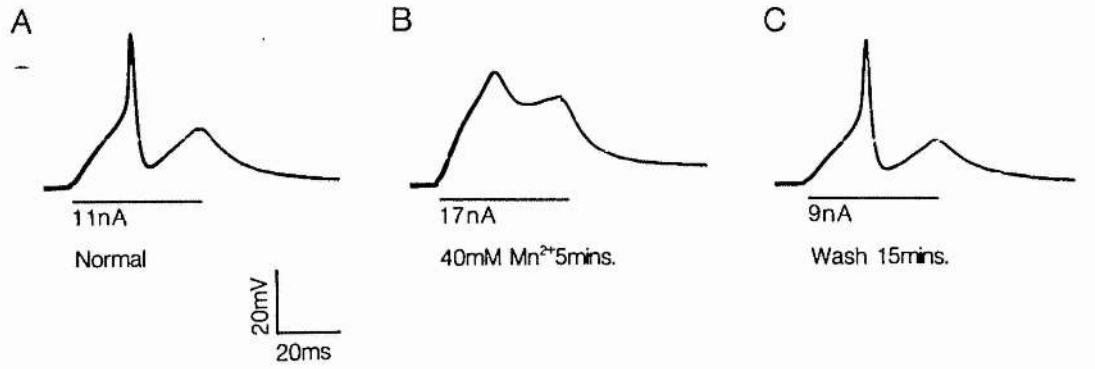


Fig. 3.331 6

The effect of Mn^{2+} saline on the carbon dioxide-induced action potential.

A. Normal carbon dioxide-induced action potential.

B. After 5mins. Mn^{2+} (40mM) treatment the action potential was completely reduced to a graded spike.

C. Restoration of the action potential after 15mins. wash with normal saline.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -66mV.

3.332 AXOTOMY.

Approximately 4-5 days after axotomy the cell body of cell 3 was able to sustain all-or-nothing action potentials in response to depolarising current pulses (Fig. 3.332 1). Action potentials were typically 50 to 70mV in amplitude and 3 to 7ms duration.

The ionic basis of the axotomy-induced action potential was investigated. The regenerative phase of the action potential was reversibly reduced but not completely abolished in Na-free saline (Fig 3.332 2). Nominally Ca-free saline reduced the amplitude and caused a broadening of the action potential but did not produce complete block (Fig. 3.332 3). Although the normal bathing saline was exchanged for one lacking calcium it is likely that sufficient Ca^{2+} could remain trapped within the infoldings of the soma membrane and between surrounding glial cells to support Ca-dependent action potentials. A four-fold increase in Ca^{2+} caused only a small increase in the action potential (Fig. 3.332 4). Mn^{2+} (40mM) reduced the amplitude of the regenerative phase and caused a broadening of the action potential (Fig. 3.332 5) similar to that observed with Ca-free saline. When Na-free saline and Mn^{2+} (40mM) were combined the action potential was reduced to a graded spike (Fig. 3.332 6). The block was more complete than that observed with Na-free saline or Mn^{2+} alone.

From these results it appears that the inward current of the axotomy induced action potential was carried mainly by sodium ions with a minor calcium ion contribution.

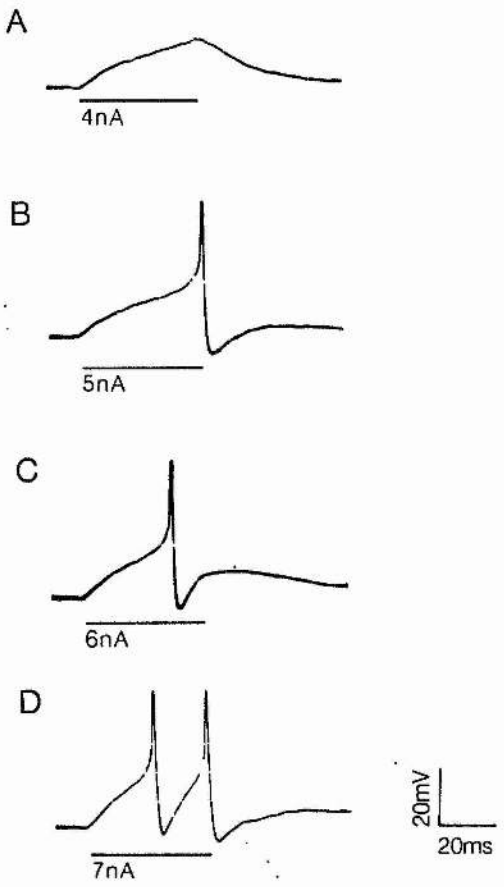


Fig. 3.332 1

The effect of axotomy on the membrane response during intracellular depolarisation.

A - D. Gradually increasing the magnitude of applied current evoked an all-or-nothing action potential with a typical duration of 3 to 7ms.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -75mV.

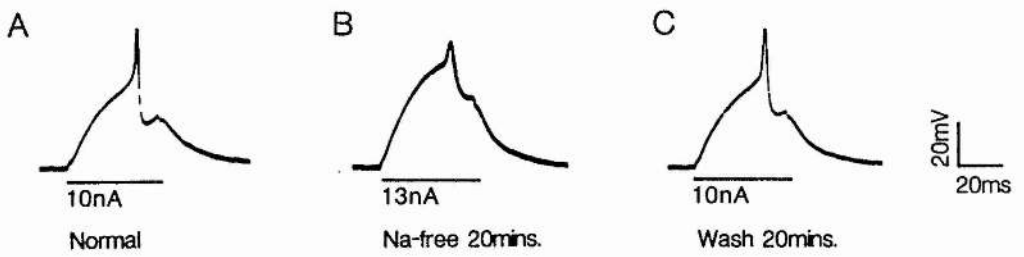


Fig. 3.332 2

The effect of Na-free saline on the axotomy-induced action potential.

A. Normal axotomy-induced action potential.

B. After 20mins., Na-saline severely reduced but did not fully abolish the regenerative phase of the action potential. The threshold for spike initiation was much higher than in normal saline.

C. Restoration of the action potential after 20mins. wash in normal saline.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -58mV.

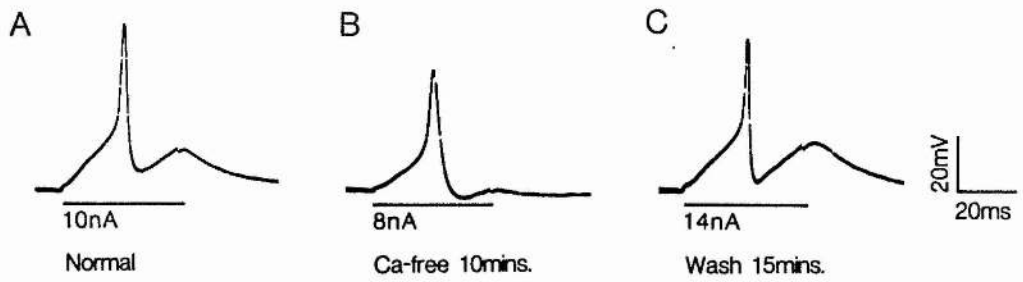


Fig. 3.332 3

The effect of Ca-free saline on the axotomy-induced action potential.

A. Normal axotomy-induced action potential.

B. After 10mins., Ca-free saline reduced the amplitude and caused broadening of the regenerative phase of the action potential.

C. Restoration of the action potential after 15mins. wash with normal saline

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -77mV.

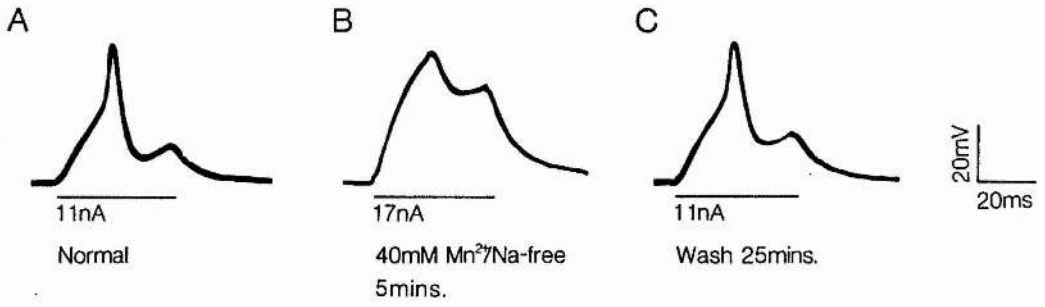


Fig. 3.332 6

The effect of Mn^{2+} in Na-free saline on the axotomy-induced action potential.

A. Normal axotomy-induced action potential.

B. After 5mins., Mn^{2+} in Na-free saline reduced the action potential to a graded spike. The combined treatments provided a more complete block than either alone could achieve.

C. Restoration of the action potential after 25mins. wash with normal saline.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -68mV.

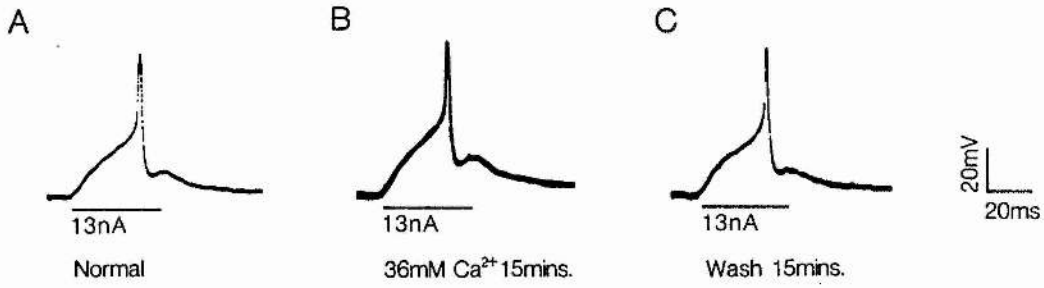


Fig. 3.332 4

The effect of high calcium saline on the axotomy-induced action potential.

A. Normal axotomy-induced action potential.

B. After 15mins., high calcium (36mM) saline had no significant effect on the action potential.

C. After 15mins. wash with normal saline

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -79mV.

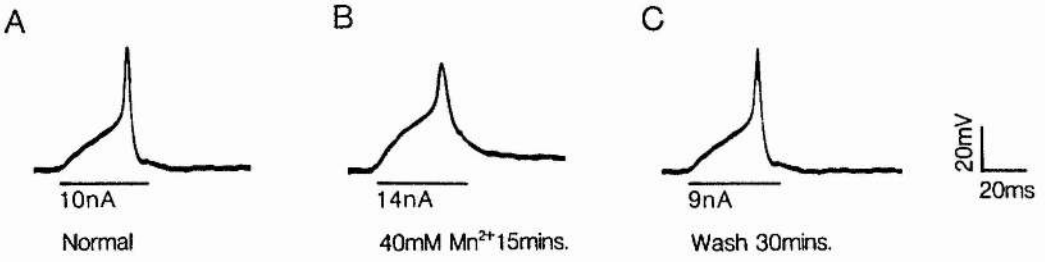


Fig. 3.332 5

The effect of Mn^{2+} saline on the axotomy-induced action potential.

A. Normal axotomy-induced action potential.

B. After 15mins., Mn^{2+} (40mM) saline caused a reduction and a broadening of the action potential. The repolarisation phase was much slower than before treatment.

C. Restoration of the action potential after 30mins. wash with normal saline.

Bar underneath represents the current pulse duration. Current intensities shown underneath. Resting potential = -64mV.

4. DISCUSSION.

The results presented here have many features in common with those recorded from the soma of cell 28 both under voltage-clamp (Thomas, 1984) and current-clamp (Pitman, Tweedle and Cohen, 1979; Pitman, 1979; 1988). Physiologically, these two motoneurones are partial synergists innervating the bifunctional coxal/depressor muscles. Cell 28 innervates muscles 177D, 177E, 178 and 179 while cell 3 innervates muscle 177C (Carbonell, 1947; Pearson and Iles, 1970). Both motoneurones have a strong calcium-dependent outward current. This study attempts to further characterise the pharmacology of the outward currents. In addition, tail currents from these motoneurones have not been previously studied. The normal electrical properties of this neurone were studied under voltage-clamp before trying to induce electrophysiological changes, though it was out of the scope of this study to examine the current response after anoxia or axotomy. This study has formed the groundwork on which further work on excitability can be based.

4.1 Normal voltage-clamp response.

Most of the research carried out on insect nerve cell bodies has focussed on receptor-mediated ion channels and agonist/antagonist interactions. Voltage-dependent ion channels have probably been neglected because the cell bodies of most insect neurones are not actively invaded by action potentials. The distinction between these two types of ion channel has become blurred since the discovery of transmitter-mediated voltage-dependent ion channels. There is currently no direct evidence, however, for their presence in the insect nervous system (Pitman, 1985). This study has focussed on the voltage-dependent ion channels of the soma of cell 3.

Sequentially depolarising cell 3 produced a series of net-

outward current responses. Since a major component of the outward current in cell 3 is apparently activated by calcium influx it should be possible to record a net inward current from these neurones. Although citrate-filled microelectrodes and externally applied TEA^+ cause the soma of some insect motoneurones to generate Ca-dependent action potentials under current-clamp conditions, (Pitman 1979), neither Thomas (1984) nor the study undertaken here have provided direct evidence of a significant inward current under voltage-clamp conditions. The reason for this may be that the inward current is masked by a relatively rapidly developing, large outward current. The inward calcium current is likely to be small as Thomas (1984) demonstrated that small intracellular increases in calcium concentration were sufficient to fully activate the g_K.

Using an external pharmacological cocktail of TEA^+ and 4-AP combined with barium to block outward K currents and to enhance inward currents through calcium channels, Christensen *et al.* (1988) were able to record an inward current from cultured embryonic insect neurones from cockroach brain. The inward current activated rapidly with maximal activation occurring around 0mV command potential, evoking a current of approximately 530pA in magnitude. Inactivation was much slower, the rate of which decreased as the command potential was depolarised. A similar cocktail of outward current blockers would be needed to effectively study inward currents in cell 3.

The net outward current I-V relationship has an N-shape similar to that originally reported in molluscan neurones (Meech and Standen, 1974; 1975). A slowly developing current is responsible for the hump in the I-V relationship which can be abolished by Cd^{2+} or Mn^{2+} and thus appears to be Ca-dependent. The negative conductance region of the cell 3 response is more pronounced and steeper in comparison to

the molluscan response. This region can be explained as follows: the command potential approaches the calcium equilibrium potential the driving force on the ion is reduced and therefore no more Ca^{2+} is able to enter the cell and activate the calcium-dependent outward current. Since this is a major component of the outward current, a decrease in the current response is initially observed. The subsequent increase in current does not therefore represent an increase in a Ca^{2+}-dependent outward current, but represents an increase in another current or currents.

The strong time dependency of the N-shape with respect to current measurements has also been observed in cell 28 of the cockroach (Thomas, 1984) and to a lesser extent in *Helix* neurones (Lux and Hofmeier, 1982a).

The peak current response seen with long duration command pulses was abolished by Cd^{2+} and is thus apparently due to the calcium-activated current component. The decay of this component is slower than its activation. These experiments indicate that the mechanism underlying the current decline may involve I_{KCa} channel inactivation or some other process involving ion redistribution. Inactivation is the rapid process by which ion channels close and are no longer able to open until inactivation is removed. The apparent inactivation observed during long duration command pulses could have been due to potassium efflux whereby potassium accumulation results in a reduction of the transmembrane K gradient with a consequent reduction in outward current carried by these ions. It is unlikely that this is entirely responsible for the decline because an outward current persists in the presence of Cd^{2+} which may represent the calcium-independent I_K. In addition, successive long duration command pulses cause the current peak to decline and eventually disappear leaving the steady-state current of the plateau region unaffected. This is,

however, a temporary loss as resting the cell for several minutes permits the return of the current peak. Instead the apparent inactivation of the peak response and its loss during repetitive depolarisations may have been due to either Ca depletion in the extracellular space or calcium accumulation within the cell. Ca^{2+} depletion is unlikely because of the high concentration of Ca^{2+} in the bathing saline. Instead, intracellular Ca^{2+} accumulation during a sustained depolarisation may feed-back on I_{Ca} and so prevent activation of I_{KCa} as in molluscan neurones (see Fig 1.223 1; Eckert, Tillotson and Brehm, 1981).

The rapid current rise times between command potentials of -50mV and -80mV suggest that very little calcium is required to activate a strong outward current. Although the experiments presented here do not directly address the question of an activation mechanism, a study undertaken on a similar cockroach motoneurone, cell 28, indicated that short periods of calcium entry were sufficient to fully activate g_K (Thomas, 1984). There are two alternative hypotheses regarding the way in which calcium activates the outward current:

1. The passage of calcium through the membrane is crucial for the activation of the potassium channel. Presumably the inward calcium channels are in close proximity or may be linked to the potassium channels in order to allow the influx of calcium ions to gate the potassium channel.
2. The increase in calcium concentration at the intracellular membrane surface causes the activation of the potassium channel.

If Ca^{2+} passing through the membrane were sufficient to activate I_{KCa}, then the appearance of citrate induced action potentials would be solely due to the increased driving force on Ca^{2+}. This is

unlikely because intracellular citrate has been demonstrated to cause an increase in input resistance in cell 28, indicating ion channel closure brought about by the suppression of an outward current (Pitman, 1979). Activation of I_{KCa} within cell 3 may, therefore, be due to intracellular accumulation of Ca^{2+} and may not be directly linked to Ca^{2+} traversing the membrane.

Symmetrical positive and negative command steps evoke a progressively large inward currrent at potentials more negative than -190mV and an enhanced outward current at potentials more positive than +110mV. The tail current reversal potential for the outward current lies between -60 and -80mV. A large hyperpolarising command step may then be sufficient to reverse the driving force on the ion responsible for the outward current such that it now enters the cell and membrane rectification breaks down. Ions entering the cell during a hyperpolarising command step could, therefore, transiently increase the intracellular concentration of that ion and so increase the driving force on the ion during subsequent depolarisation which would account for the enhanced current response during large depolarising command steps.

4.2 Pharmacology of the outward currents.

The pharmacology of the outward currents was investigated in terms of the three main potassium currents extensively reported in molluscan neurones, I_A, I_K and I_{KCa} (Thopson, 1977: Adams, Smith and Thompson, 1980). There are a number of compounds which have been reported to specifically block one or more of the potassium currents but their action is often not entirely specific.

4.21 Organic and inorganic calcium blockers.

In this preparation verapamil, at low concentrations, did reduce the calcium-dependent hump in the I-V relationship. Verapamil and related compounds have been used therapeutically as calcium antagonists in the treatment of supraventricular cardiac arrhythmias and angina pectoris (Fleckenstein, 1977). More recently low concentrations (10-50μM) of verapamil, D600 and diltiazem have been reported to directly and selectively block I_{KCa} independently of blocking I_{Ca} in *Helix* neurones (Gola and Ducreux, 1985). Under current-clamp, external application of these compounds caused a broadening of the action potential and, under voltage-clamp, they caused an 80% suppression of I_{KCa} within 3mins. while the inward current remained relatively stable. This contrasted with the mechanism of cadmium blockade which exhibited an almost parallel reduction in I_{Ca} and I_{KCa}. The inward current was eventually reversibly reduced whereas I_{KCa} blockade was irreversible. On the other hand intracellular injection of D600 now caused a parallel decline in I_{Ca} and I_{KCa} suggesting that I_{KCa} blockade was secondary to I_{Ca} blockade. Gola and Ducreux (1985) concluded that D600 when externally applied, was blocking the K_{Ca} channel on the external membrane surface.

After prolonged exposure to verapamil the I-V relationship in cell 3 differed from that obtained with Cd^{2+} or Mn^{2+} blockade in

that currents in the lower arm region appeared to be further reduced in a manner similar to that after TEA^+ treatment. If verapamil were exerting a similar effect to TEA^+, however, application of TEA^+ in the presence of verapamil would not cause further decline in the outward current. This remains to be explored. Although verapamil, like TEA^+, is apparently able to block a large part of the outward current it is unlikely that verapamil would enhance excitability in the same way as TEA^+ because of its action as a calcium blocker thereby preventing the inward current.

Alternative explainations for the action of verapamil are that either the block observed after Cd^{2+} and Mn^{2+} is not complete or verapamil blocks a Ca-independent current component as well as the Ca-dependent current. There is evidence to support the latter proposition from work carried out on other preparations. Although the main action of organic calcium blockers is to block the transmembrane movement of Ca^{2+}, in molluscan neurones verapamil also suppresses I_A and I_K (Kostyuk, Kristal and Doroshenko, 1975). Kostyuk *et al.* (1975) proposed that in the molluscan system verapamil behaves like internally applied TEA^+ in depressing the steady-state component of I_K. Ishikawa *et al.* (1985) observed that high concentrations of the benzothiazepine type blocker, dilitiazem (73uM), depolarised guinea-pig smooth muscle cells with an accompanying increase in input resistance. This suggested that diltiazem was blocking other channels as well as, or instead of, calcium channels. The phenylalkylamine type blocker, D600, inhibited an outward potassium current in Purkinje fibres (Kass and Tsien, 1975). Diltiazem, D600 or nisoldipine were also observed to block the outward current in frog atrial cells (Hume, 1985). D600 was reported to block I_H in *Helix* neurones (Meech and Thomas, 1987) and verapamil blocked what is now known to be I_H in perfused *Lymnaea*

neurones (Byerly and Hagiwara, 1982). In view of the evidence of this other work it is likely that verapamil does not specifically block I_{KCa} in this preparation.

An experiment to test whether verapamil blocks a Ca-independent current in cell 3 would be to apply moderately high concentrations of Cd^{2+} or Mn^{2+} to the cell in order to rapidly abolish the hump and then apply verapamil and to see if there was any further decrease in the I-V relationship, assuming that Cd^{2+} and Mn^{2+} had caused a complete block of the calcium-dependent current.

An additional effect of verapamil treatment in cell 3 was to cause apparent increased inactivation of the remaining current which was more pronounced with long duration (500ms) command pulses. Unlike the inorganic Ca blockers which competitively block Ca binding sites, verapamil has been reported to exert its blockade by altering channel kinetics. For example, external verapamil has been reported to increase the inactivation of I_K in *Helix* neurones. These organic blockers are membrane permeant in cardiac muscle (Hescheler, Pelzer, Trube and Trautwein, 1982) and therefore may also be exerting their actions intracellularly, either on the inner membrane surface or on cytosolic stores e.g. mitochondria or ER.

Verapamil was the only organic calcium antagonist to be tested on cell 3. One would expect the related phenylalkylamine blockers D600 and D888 (desmethoxyverapamil) to also block the calcium-dependent hump. Indeed, D600 was successfully used by Thomas (1984) to block I_{KCa} in cell 28. There was, however, no mention of D600 non-selectively blocking other currents.

4.22 Apamin.

Provisional results indicate that apamin was not able to suppress the I_{KCa} in cell 3. Either the calcium-dependent outward current is

insensitive to apamin or the apamin solution was ineffective. From the literature, the apamin-sensitive I_{KCa} corresponds to a small conductance channel (10-20pS) which is TEA^+-insensitive in muscle preparations (Romey and Lazdunski, 1984); Blatz and Magleby, 1986a). The results obtained in this study on cell 3 indicate that the I_{KCa} is apamin-insensitive and TEA^+-sensitive and therefore conforms to the aforementioned I_{KCa} category. It would be interesting to test the scorpion toxin charybdotoxin which is selective for the large conductance I_{KCa} (Miller, Moczydlowski, Latorre and Phillips, 1985). A study by Dunbar and Pitman (1985) has already recorded low conductance outward current channels of 5.6pS and 11pS in the soma of cell 28, though it was not determined if either of these channels were activated by calcium. Further experiments are necessary to fully explore a possible role for apamin in the insect nervous system.

In frog skeletal muscle, apamin-receptor binding appears to be dependent upon extracellular Ca^{2+} concentration (Traoré, Cognard, Potreau and Raymond, 1986). Apamin added to a low Ca^{2+} saline caused a 50% decrease in the outward current which was more pronounced than block in the normal saline (33% block), whereas apamin in high Ca^{2+} saline only produced a 20% reduction. Synaptosomal studies also indicate that apamin binding is dependent on external cation concentration. Low K^+ or rubidium (Rb^+) concentrations (10μM to 5mM) increase mono [^{125}I] iodoapamin binding, whereas higher concentrations (>5mM) decrease affinity (Hugues, Duval, Kitabgi, Lazdunski and Vincent, 1982). The interpretation was that there are two binding sites: site 1 is the high affinity binding site for K^+ and Rb^+ which is distinct from site 2, the apamin receptor. Cation occupation of site 1 induces a conformational change which encourages apamin binding whereas occupation of site 2 competitively inhibits

apamin binding. The cations may compete with the positively charged apamin side chains of Arg 13 and 14 which are thought to participate in receptor binding, presumably to an anionic site (Hugues *et al.*, 1982).

During the experiments described here apamin was dissolved in normal saline (pH 7.2) and stored frozen in small aliquots to be defrosted when required. In future experiments Bovine serum albumin should be added to the stock solution to reduce the likelihood of apamin adsorbing onto the containers or experimental chamber. A cautionary note is that in some laboratories the efficacy of some batches of apamin obtained from SIGMA appear to be better than others which were only about 50% pure (Blatz, personal communication). This was also supported by correspondence with Guy Raymond who suggested that results with apamin strongly depend on the purity of the sample and recommended apamin from an American company called "SERVA". In addition, he suggested that concentrated frozen aliquots of apamin are very stable but when stored at lower concentrations some loss of activity was noted, probably due to surface adsorption or agglomeration.

4.23 TEA^+.

TEA^+ (Br^-) was used in preference to TEA^+ (Cl^-) which has been reported to contain an impurity, triethylamine, which increases the pH of the cytoplasm (Zucker, 1981). Commercial TEA^+ is synthesised from triethylamine which is a weak base. Triethylamine has been employed as a pharmacological tool in alkylising the cytoplasm and observing the effects of pH perturbation in snail neurones (Thomas, R. C., 1984). Zucker (1981b) reported that high intracellular pH resulting from ammonia treatment reduced the calcium buffering ability in molluscan neurones. The net effect was a large increase

in intracellular free Ca^{2+} concentration during calcium influx brought about by electrical activity. This would obviously have important consequences for neural properties.

In this preparation externally applied TEA^+ appeared to block the calcium-dependent current. Although classically TEA^+ has been reported to selectively block I_K in different preparations, it has different effects on other potassium currents depending on its concentration and whether it is applied internally or externally to the membrane (see section 1.221). TEA^+ has been reported to block a significant portion of I_{KCa} in a number of preparations e.g. *Helix* neurones (Meech and Standen, 1975), *Aplysia* neurones (Hermann and Gorman, 1981; Chad, Eckert and Ewald, 1984; Dietmer and Eckert, 1985), reconstituted I_{KCa} in planar bilipid layers (Latorre, Vergara and Hidalgo, 1982), rabbit portal vein smooth muscle (Inoue, Kitamara and Kuriyama, 1985) and an anterior pituitary cell line (Wong and Adler, 1986). In contrast, I_{KCa} has been reported to be TEA^+-insensitive in neurones of nudibranch molluscs (Aldrich, Getting and Thompson, 1979; Thompson, 1977). In addition, both TEA^+-sensitive and TEA^+-insensitive I_{KCa} components can occur in the same molluscan neurone (Deitmer and Eckert, 1985).

Abolition of the hump in the I-V relationship by cadmium (1mM) and subsequent application of TEA^+ caused a further reduction in outward current. This suggests that TEA^+ also blocks a calcium-independent current in addition to a Ca-dependent component. The relative sensitivities of the two current componnets to TEA^+ were not established. The profound 'blocking action of TEA^+ on the outward currents of cell 3 accounts for the appearance of soma excitability during TEA^+ treatment under current-clamp. There is a question as to whether TEA^+ is blocking a Cd^{2+}-insensitive current component or enforcing a more complete block on the same current as Cd^{2+}. The

latter is unlikely since increasing Cd^{2+} concentration from 1mM to 10mM did not block the lower arm currents to the same extent as TEA^{+}. Also, cadmium even at concentrations of 1mM, abolised the hump in the I-V relationship which is characteristic of the Ca-dependent current component. The calcium-insensitive component has been referred to as I_K because of its sensitivity to TEA^{+}.

Although activation of I_K is not dependent on Ca^{2+} influx into the soma, external Ca^{2+} can affect the properties of potassium channels. Some of these alterations have in the past been interpreted as indirect surface charge actions where charge screening causes an apparent voltage-shift in voltage-dependent parameters (Frankenhaeuser and Hodgkin, 1957). Recent investigations, however, have shown that calcium may also play an important specific role in the normal functioning of the delayed rectifier K^{+} channel (for review see Begenisich, 1988). Gilly and Armstrong (1982) proposed that calcium bound near the external surface of the K-channel protein stabilises the channel in the closed configuration. The channel cannot open until Ca^{2+} leaves the binding site. Further work by Armstrong and Lopez-Barneo (1987) showed that complete removal of external Ca^{2+} abolished the K^{+} current and was accompanied by an increase in apparent current leak thought to be due to a conformational change leading to the loss of channel ion selectivity. It is clear from these observations that Ca^{2+} is not only essential for the normal functioning of I_{KCa} but also for the normal functioning of I_K.

4.24 Aminopyridines.

If a current component similar to I_A made a significant contribution to outward currents in cell 3 one would expect to see a current transient when the membrane potential was stepped from a holding

potential of -90mV to potentials less positive than -30mV. There was, however, no evidence to indicate the presence of a current analogous to I_A in cell 3 or in a similar cockroach motoneurone, cell 28 (Thomas, 1984). In contrast cultured embryonic neurones from cockroach brain have been reported to exhibit a 4-AP-sensitive outward current (Christensen *et al.*, 1988). The order of magnitude of I_A in molluscan neurones ranges from one to several hundred nA (Connor and Stevens, 1971b; Thompson, 1977). Although the net outward current response measured in this study usually reached the uA range and the expected I_A response would be an order of magnitude less, a response of this size would have been visible. Thus, I_A, if present at all makes only a small contribution to the net outward currents in adult cell 3.

If a fast transient current were present, perhaps the experimental conditions were not optimal to observe this current component. In the experiments presented here a holding potential of -90mV may not have been sufficiently negative to remove the inactivation of I_A. More negative holding potentials were not employed with the single command step regime in order to reduce the possibility of cell damage. In order to fully investigate the possibile presence of a transient current, a double pulse command step regime is required. This would enable a short conditioning hyperpolarising (greater than -90mV) step to be given directly followed by a test pulse to various depolarised potentials. Unfortunately it was not possible to generate such command steps at the time of these experiments. For molluscan neurones the peak amplitude of I_A depends upon the conditioning command voltage and the following test step voltage (Connor and Stevens, 1971b). This technique combined with kinetic and pharmacological investigation would provide a more thorough search for I_A in cell 3.

Although the aminopyridines did not block an early current under voltage-clamp they did shift the voltage-dependency of the hump activation toward more negative potentials. This represents a hastened rise of the slowly developing current. The mechanism of this is unclear.

Although 4-AP has been reported to induce all-or-nothing action potentials in the locust FETi (Goodman and Heitler, 1979), application of aminopyridines to cell 3 did not enhance excitability in cell 3 under current-clamp. In fact in current-clamp recordings both 4-AP and 3,4-DAP caused a reduction in the amplitude of the voltage response produced by current pulses of standard amplitude with the eventual loss of the oscillatory response. This represents a large conductance increase. Aminopyridines also caused a progressive slow depolarisation which was also observed in the locust FETi (Goodman and Heitler, 1979). This depolarisation could be accounted for at least in part by an increase in non-specific leakage current. It is therefore possible that the leakage current has a calcium component which is able to activate g_{KCa} and, under voltage-clamp, produce the shift in the I-V relationship representing an enhanced outward current for a given command potential. An enhanced outward current may effectively dampen the oscillatory response. The slow depolarisation may also contribute to the inactivation of an inward conductance and thereby suppress excitability.

Although at low concentration (<2mM) the aminopyridines and their derivatives are selective for I_A, when externally applied to molluscan neurones (Thompson, 1977; Hermann and Gorman (1981a) at concentrations of 5mM or greater, aminopyridines affect other potassium conductances. When inward Na^+ and Ca^{2+} currents were blocked, 4-AP blocked the remaining I_K. In addition, 4-AP increased

the I_{KCa} evoked by intracellular ionophoretic injection of Ca^{2+} i.e. caused a g_K increase (Hermann and Gorman, 1981a). This was observed as an increase in outward current response between potentials of -70mV and +50mV. Similar changes were occasionally seen in this preparation.

4-AP is a weak base and has been reported to increase intracellular pH and buffering capacity in snail neurones in response to acid injection (Szatkowski and Thomas, 1987). Such intracellular alkalysation could, in principal, affect the calcium buffering capacity of the cell (Moody, 1984). Hermann and Gorman (1981a), however, demonstrated that this was not the case by measuring intracellular Ca^{2+} concentration with arsenazo III during application of 4-AP. They suggested that aminopyridines directly affected the activation of the g_K by Ca^{2+}.

4.25 Chloride ion contribution.

Reducing the external chloride concentration clearly had a profound effect in increasing the rate of activation of the slowly developing current. Low chloride saline also caused an overall reduction in the net outward current. Chloride ions may then have a direct role in carrying the outward current or alternatively affect the conductance of another ion.

The simplest explanation is that during depolarisation chloride enters the cell and a net inward flow of negatively charged ions is qualitatively the same as a net outward flow of positive ions which constitutes an outward current. This does, however, assume that the equilibrium potential for chloride in this cell is equal to, or more negative than, the holding potoential of -70mV. There is good evidence from current-clamp experiments that the chloride equilibrium potential in insect neurones is more negative than the normal resting potential. Gamma aminobutyric acid (GABA) hyperpolarises DUM cells

(Kerkut, Pitman and Walker, 1969a: 1969b) which could be reversed by injecting chloride into the cell body (Pitman and Kerkut, 1970).

Apart from the direct involvement of chloride ions in carrying charge, chloride may be acting indirectly on the conductance of another ion i.e. chloride entering the cell activates an outward potassium current (I_{KCl}). Thus the outward current generated by the influx of chloride ions would itself produce further outward current by activation of the potassium current. There is, however, no precedence for a chloride-activated potassium current in excitable cells. One could speculate that if there is an I_{KCl} in this neurone it may correspond to the TEA^+-sensitive/Cd^{2+}-insensitive portion of the outward current. Much more work is required to investigate the nature of the chloride contribution in cell 3.

4.3 Properties of the tail currents.

Command pulses of 50ms or greater were employed in the characterisation of the net outward currents. The tail current relaxation at the end of the command step was inward for command pulse durations greater than 50ms. On closer examination the tail current I-V relationship for command pulses greater than 20ms had a complex M-shape. The inward dip of the M-shape could be due to:

i.) an increase of an inward current,

ii.) a reduction of an outward current or

iii.) a combination of both mechanisms.

The first possibility is unlikely as the lowest point of the dip occurred around a command potential of +60mV which is far too positive for the inward dip to be explained by an increase in inward current as one would expect a cation conductance to be approaching its equilibrium potential which would in turn reduce the driving force on that ion and prevent ions entering the cell.

The second possibility is a reduction of an outward current due to a shift in E_K to such a degree that it becomes more positive than the holding potential therefore K^+ will enter the cell through channels which remain open at the end of a command pulse to produce an inward tail current. Hodgkin and Frankenhauser (1956) proposed that at the end of a command pulse ions will have accumulated in the periaxonal space. There is good evidence for the possibility of extracellular ion accumulation in the insect preparation. Histological examinations of insect neurone cell bodies have shown that the neural membrane is highly infolded (Holmgren, 1900; Hess, 1958). The finger-like processes of the inner glial layer can frequently penetrate the neurone cytoplasm to form networks of canaliculi called the trophospongium (Holmgren, 1900) which would

restrict extracellular space and facilitate ion accumulation. Thomas (1984) calculated a membrane capacitance of 10 $\mu F/cm^2$ for cell 28 which is high for the apparent surface area of the cell and further reflects the considerable membrane infoldings.

The effect of accumulation on the tail currents would be most apparent when the outward currents are large. The peak of the hump in the outward current I-V relationship is likely to reflect the inward dip of the tail current I-V relationship. Furthermore, as the outward currents rise in the upper arm of the curve this causes the tail currents to become inward again.

Superimposed on this trend is the effect of increasing the command pulse duration and thus increasing the amount of current flowing which in turn will elevate K^+ accumulation in the extracellular space. Consequently the whole M-shape will be forced more inward.

Further observations with double command steps showed that increasing the pulse (I) duration from 10 to 50ms caused a positive shift in reversal potential representing either a two-fold decrease in anion concentration or a two-fold increase in cation concentration. Although there is evidence for a chloride contribution, potassium ions are most likely to be the major outward current carrying ion since TEA^+ was able to dramatically suppress the outward current. Altering the external K^+ concentration between 3.1 and 15mM, however, had a small and inconsistent effect on the tail current reversal potential. There was certainly not the response predicted by the Nernst equation. If K^+ ions do carry most or all of the outward current, the insensitivity to external K^+ could be accounted for by restricted exchange between external solutions and the extracellular space which is consistent with the effects of pulse duration as discussed above. Thomas (1984) measured the reversal

potential of the current elicited by a standard Ca injection into the soma of cell 28. This study showed that changes in external K^+ concentrations only produced a shift in the reversal potential predicted by the Nernst equation at concentrations above 10mM. On the basis of these observations it would be necessary for future experiments to determine the K^+-dependency of the tail current reversal potentials use external K^+ concentrations between 10 to 30mM. Ultimately patch-clamping would overcome the problems of accumulation and, depending on the patch configuration employed, it would enable the internal or external ion environment to be manipulated.

4.4 Summary of voltage-dependent currents in the soma of cell 3.

In conclusion, Fig 4.4 1 shows a diagrammatic representation of identified and putative voltage-dependent currents and their associated pharmacological agents in the soma of cell 3. Although there is no indication of the relative densities of the current channels, I_K and I_{KCa} contribute a major portion of the outward current while chloride ions may make some contribution. This scheme describes the situation so far. The identification of other receptor-linked channels has not been discounted and is in itself a large and complex field of research beyond the scope of this investigation.

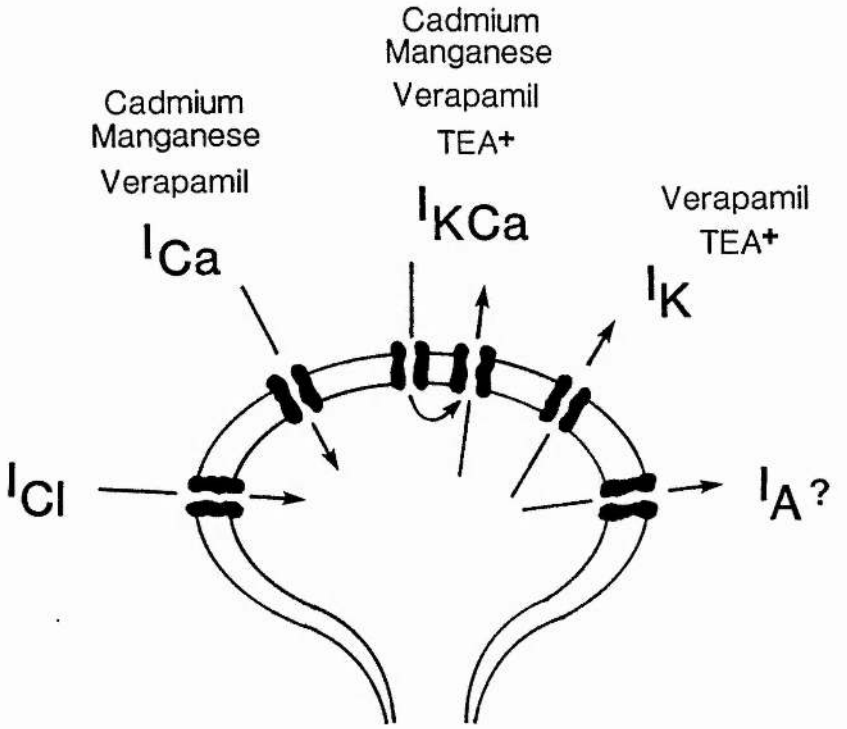


Fig. 4.4 1

A diagrammatic representation of identified and putative currents and their associated pharmacological agents in the soma of cell 3 of the cockroach (*Periplaneta americana*).

4.5 Changes in excitability under current-clamp.

4.51 Normal current-clamp response.

The dampened membrane voltage oscillations observed during current injection into insect neurones have also been reported in crustacean muscle (Fatt and Katz, 1953; Atwood, 1967) and *Paramecium* (Naitoh, Eckert and Friedman, 1972). Crustacean muscle oscillations, unlike insect motoneurone oscillations, had an inflection on the rising phase suggesting a regenerative current response. The insect response reached a more steady membrane voltage for the duration of the pulse. This could indicate a regenerative inward current followed by a rapid, strong outward current (Pitman, 1979). From the voltage-clamp data the dampening of the oscillations could be due to the progressive activation of I_{Kca} by Ca^{2+} entering the cell together with the activation of the Ca-independent component.

4.52 Acute changes in excitability.

The soma of cell 3 can be acutely enabled to support all-or-nothing action potentials by external application of TEA^+ or intracellular injection of citrate ions. Both treatments produce calcium-dependent action potentials but they are markedly different in shape (Pitman, 1979). The TEA^+-induced action potential is much broader than the citrate-induced action potential. This is not surprising because the voltage-clamp data demonstrates that TEA^+ has a profound blocking action on the outward currents and therefore repolarisation is likely to occur either much more slowly by the remaining unblocked currents or by some other mechanism e.g. calcium inactivation of calcium currents (Eckert *et al.*, 1981). On the other hand intracellular citrate is thought to chelate a proportion of the free intracellular Ca^{2+}, which would have two important consequences:

i. The transmembrane gradient for Ca^{2+} would be increased and therefore the driving force on the Ca^{2+} entering the

cell would be greater and depolarisation would evoke a larger Ca current.

ii. The calcium-dependent outward current will be indirectly reduced due to a decreased availability of free Ca^{2+} at the inner membrane surface.

Citrate effectively lowers the baseline from which calcium entering the cytoplasm activates I_{KCa} therefore I_{KCa} was still able to be activated and take part in repolarisation, albeit to a lesser extent than normal. Further evidence for the role of I_{KCa} in spike repolarisation comes from the current-clamp data where Ca-free saline and Mn^{2+} saline cause broadening of the axotomy-action potential. In fact Mn^{2+} treatment can often enhance the amplitude of the largely sodium-dependent action potential presumably by blocking Ca^{2+} activation of I_{KCa} and so delaying repolarisation.

4.53 Chronic changes in excitability.

Chronic changes in the excitability of cell 3 brought about by axotomy or carbon dioxide treatment produced action potentials which were sodium- and calcium-dependent, though the relative contributions appear to be different with the two treatments. Axotomy gave rise to largely Na-dependent action potentials with a smaller calcium contribution, while calcium apparently made a larger contribution to carbon dioxide action potentials. These findings contrast with cell 28 where colchicine (Pitman, 1975), axotomy (Pitman, Tweedle and Cohen, 1972) or anoxia (Pitman, 1988) produced largely sodium-dependent action potentials. Under normal conditions both cells produce similar oscillatory responses to depolarisation under current-clamp and have similar I-V relationships under voltage-clamp. Presumably this difference between cell 3 and cell 28 can be attributed to a different density of current channels induced by

chronic changes in excitability.

The main reason for choosing cell 3 for this study on excitability was because nerve 4, containing the axon of cell 3, is small and contains almost entirely motoneurone axons (Dresden and Nijenhuis, 1958). This removes the problem of deafferentation causing possible secondary effects in excitability.

Axotomy and anoxia have been clearly shown to enhance the electrical properties of cockroach motoneurones under current-clamp. The appearance of a regenerative inward current may in principle be directly observed under voltage-clamp, allowing a more complete analysis of changes observed under these conditions.

4.6 Comparison of an inexcitable soma with an excitable soma.

A group of insect neurones called DUM cells (Hoyle *et al.*, 1974) normally exhibit overshooting action potentials (Callec and Boistel, 1966; Kerkut *et al.*, 1968). The DUM cells appear to have symmetrical dendritic branching patterns (Hoyle *et al.*, 1974). Most of the DUM cells contain and release the neuromodulator, octopamine, at the neuromuscular junction (NMJ) (Evans and O'Shea, 1977; 1978). Subsequent investigations in the locust, however, showed that not all of the DUM cells were unpaired, and some DUM cells were intra-ganglionic interneurones (Hoyle, 1978).

The action potentials from DUM neurone cell bodies in the cockroach 6th abdominal ganglion were shown to be abolished in Na-free saline (Jégo, Callec, Pichon and Boistel, 1970). In the locust, action potentials from DUMETi were also blocked by Co^{2+} or La^{3+} indicating a calcium contribution in addition to a sodium contribution (Goodman and Heitler, 1979).

Under voltage-clamp the current response from motoneurone 28 and a DUM neurone of the cockroach had a similar N-shape I-V relationship (Thomas, 1984). With DUM cells there was, however, a net-inward current which was most evident at a command potential of -30mV and which persisted in Ca-free saline presumably due to a sodium contribution. The outward currents recorded from DUM cells were almost an order of magnitude smaller than cell 28 in accordance with a smaller cell body size. The major difference between the two responses was that the negative conductance region of the DUM cell response occurred at more negative potentials (between +60 to +70mV), compared to +100mV for cell 28, though the physiological significance was unclear. From this study it appears that the main difference in terms of voltage-dependent ion channels between an inexcitable and excitable cell body is the presence of sodium channels capable of

generating a net inward current.

Single channel recordings from insect neurones indicate a difference in the classes of outward unitary current in these two nerve types (Dunbar and Pitman, 1985). Dunbar and Pitman (1985) identified at least three classes of outward unitary currents recorded from the soma of DUM neurones, with conductances of 11, 34 and 110pS. The two lower conductance channels were demonstrated to conduct K^+. The inexcitable soma of cell 28 also had the 11pS channel and in addition a smaller 5.6pS channel, but lacked the 34 and 110pS channels. It appears, therefore, that the differences in excitability shown by different classes of insect neurone may depend not only upon the type of inward channel present but also upon the differences in populations of outward current channel.

4.7 Physiological and technical significance of an inexcitable soma.

Insect neuronal somata are situated at the periphery of the ganglion. The cell bodies of insect neurones are devoid of synapses. The dendritic tree branches off the unipolar axon at some distance from the cell body and arborises within the central neuropile where input and output synaptic connections are made with other neurones. Why then are these voltage- and ion-gated channels present in the soma when there is no apparent functional synaptic role for them? Perhaps an inexcitable cell-body i.e. one that cannot normally sustain action potentials, would ensure that action potentials travel orthodromically in that the soma may otherwise act as a "sink" and cause an inappropriate second rebound impulse to follow the initial one. The combination of a relatively high resting potential (between -70 and -75mV) and a large (in the μA range) rapid outward current elicited on depolarisation maintains the cell body in an inexcitable

state. Despite the lack of synaptic contact it would be interesting to test the effect of transmitter substances e.g. acetylcholine, dopamine, GABA, octopamine, upon the I-V relationship. Voltage-dependent ions observed under voltage-clamp may be influenced by a neuromodulators and/or neurohormones leading to changes in the electrical properties of the soma.

The question which faces pharmacologists is, whether the electrical characteristics of a neurone cell body is in any way reflective of the electrical properties within the distant synaptic regions. Evidence from crayfish shows that axotomy-induced action potentials in the efferent flexor inhibitor neurones were also accompanied by increased excitability in the neurites (Kuwada and Wine, 1981). There was also evidence for an increase in excitability in the neuropile of the locust DUMETi after axotomy (Goodman and Heitler, 1979). This theoretical extrapolation is an important consideration because the accessibility and size of the soma provide attractive advantages for electrophysiological and pharmacological investigation. In addition, the soma can be acutely isolated by surface aspiration (Pitman, 1988).

4.8 Future research direction.

The objective of this thesis was to characterise as far as possible the voltage-dependent net outward currents. An extension of this work would be to characterise the single channel properties underlying these macroscopic currents. So far there have been only two reports of single channel recordings from insect neurones in the literature. The first report by Dunbar and Pitman (1985) provided the first clear evidence for different channel populations underlying the physiology of electrically excitable (DUM cell) and inexcitable (cell 28) motoneurones. The second major report by Chistensen *et al.* (1988) used cells from cultured embryonic cockroach brains and found

unitary conductances ranging from 15 to 110pS (15, 35, 74 and 110pS). Though to what extent the electrical properties of cultured embryonic neurones relate to the properties of *in situ* adult neurones remains to be investigated. The study by Dunbar and Pitman has the advantages of working with an identified neurone and can thus relate experimental findings to macroscopic currents and cell physiology. In addition, results from the same identified cell in different preparations can be more readily compared.

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APPENDIX I. COMPOSITION OF INSECT SALINES.

SALINE.	4M NaCl (ml)	1M KCl (ml)	1M $CaCl_2$ (ml)
Normal	53.5	3.1	9.0
Na-free	*1	3.1	9.0
Ca-free	55.5	3.1	*2
18mM Ca	50.1	3.1	18.0
36mM Ca	43.5	3.1	36.0
47mM Cl	*3	3.1	9.0
1.5mM K	54.0	1.55	9.0
6.2mM K	52.8	6.20	9.0
12.4mM K	51.3	12.4	9.0
24.8mM K	48.1	24.8	9.0

*1 Na-free saline contained 248.4 ml of 1M TRIS-HCl.

*2 4.5 mM Ca saline was achieved using a 1:1 dilution of normal saline and Ca-free.

*3 47mM Cl saline contained 188.1 ml 1M NaAc and 25.9 ml 1M NaCl.

Stock solutions were made up to approximately 950ml with Milli Q pure water before TES (N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid) buffer (2.292g) was added. This strongly acidic solution was adjusted to pH 7.2 by adding sodium hydroxide (1M) and the pH continuously monitored using a temperature compensated pH meter (PHM 85 Precision pH Meter, Radiometer Copenhagen). Solutions were then made up to their final volume of 1000ml and the pH re-checked. For electrophysiological recordings, sucrose (100mM) was added to the salines in order to prevent osmotic swelling of the cell.

APPENDIX II,

ELECTRONIC AND BLOCK CIRCUIT DIAGRAMS.

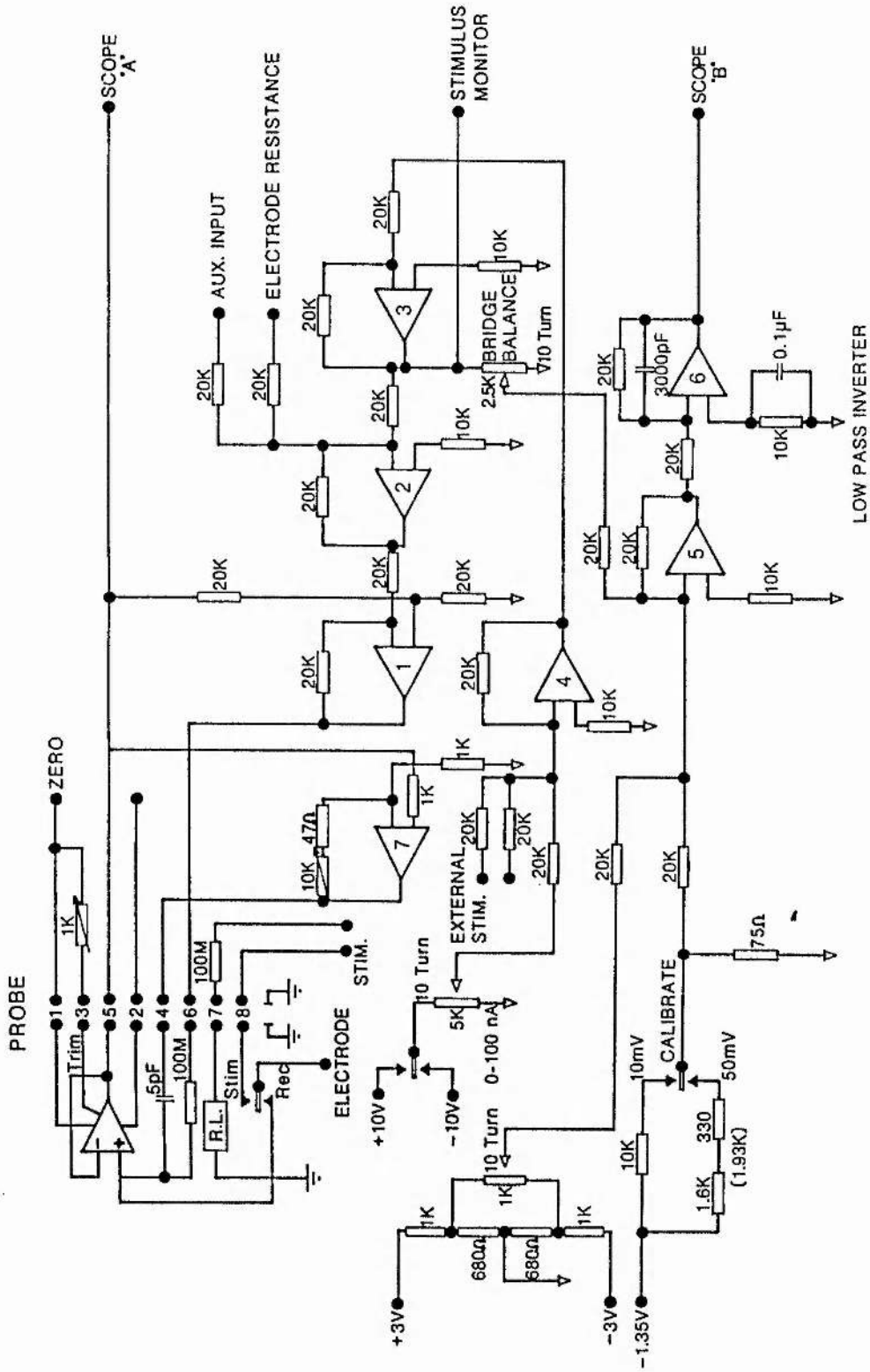


Fig. IIA

Circuit diagram of the Eastwood Intracellular Recording System including the probe configuration containing the reed relay switch enabling recording and passing current through the microelectrode.

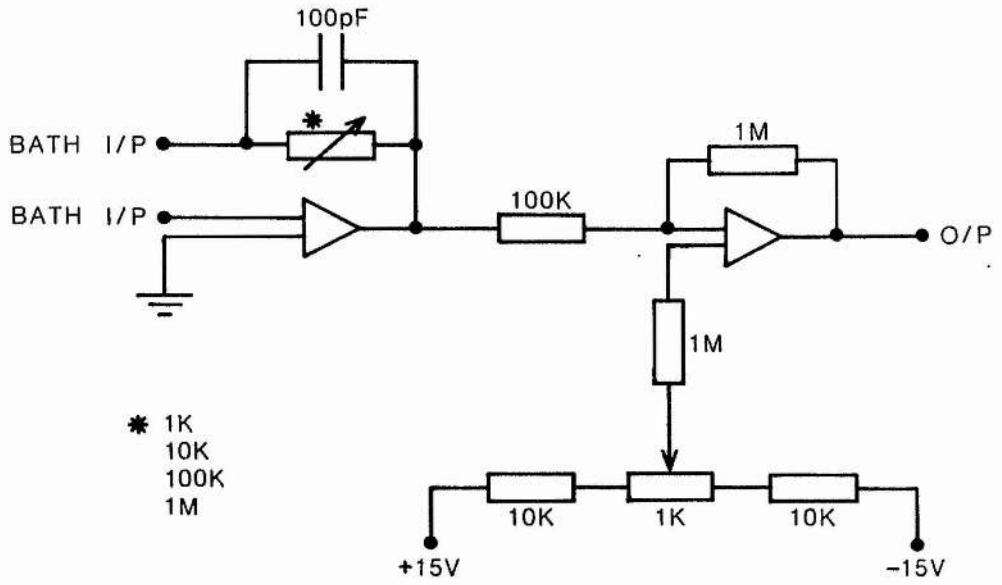


Fig. IIB

Circuit diagram of the current monitor showing a virtual earth system with a switched variable gain and an offset.

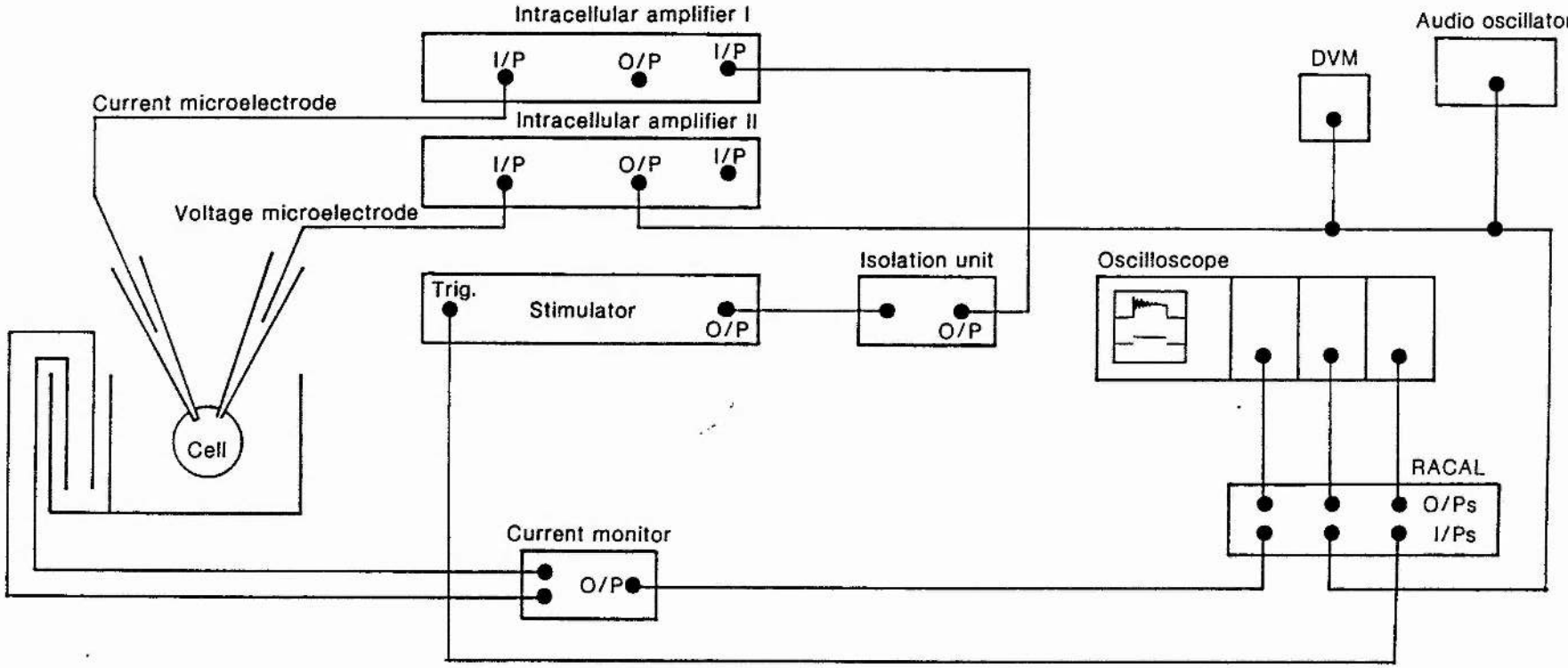


Fig. IIC

Schematic block diagram of the current-clamp recording configuration.

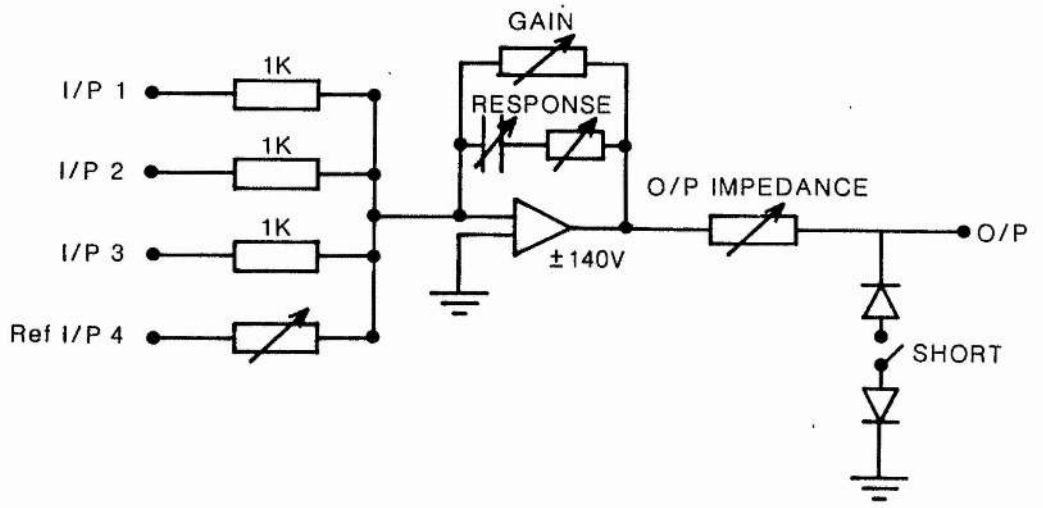


Fig. IID

Simplified circuit diagram of the voltage-clamp amplifier. I/P 1 is from the output from the differential amplifier between the membrane potential and the bath potential. I/P 2 is from the output from the laboratory designed interface.

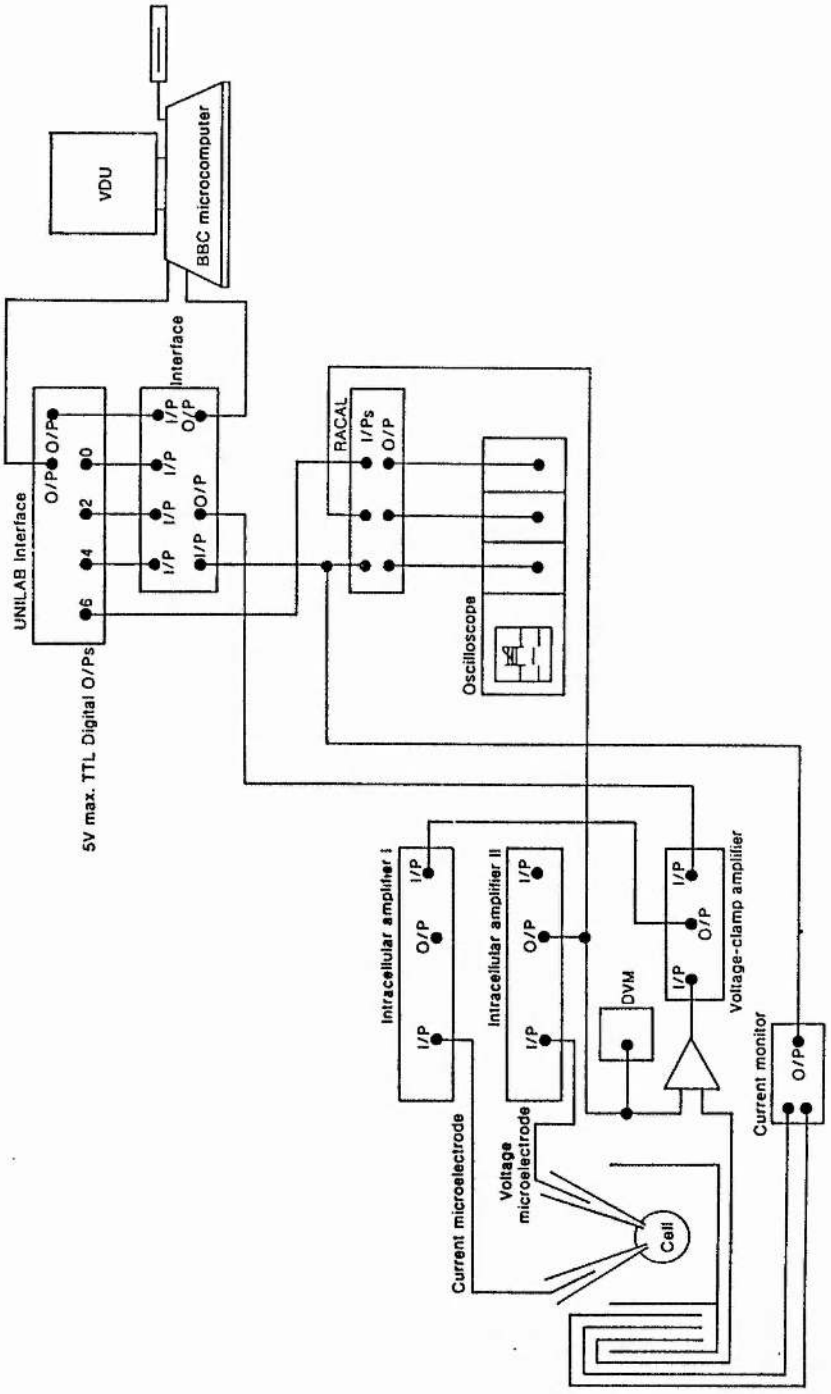


Fig. IIE

Schematic block diagram of the voltage-clamp recording configuration.

N.B. The bath reference electrode input to the differential amplifier (asterisk) should be positioned in the preparation chamber and not in the electrode chamber as illustrated here. The agar bridge between preparation and electrode chambers (not shown) offers a series resistance between the voltage microelectrode and the bath electrode which may offset the output of the differential amplifier.

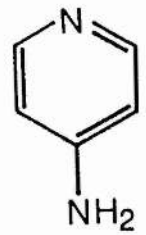
APPENDIX III DRUGS.

IIIA Origin of drugs.

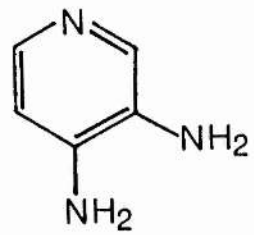
Apamin	Sigma
4-aminopyridine	BDH
3,4-diaminopyridine	Koch-Light Laboratories Ltd
Cadmium chloride	BDH
Calcium chloride	BDH
Canada balsam	BDH
Citric acid	Sigma
Creosote	BDH
Ethanol (96/100%)	BDH
Gum acacia	BDH
Hexamminecobaltic chloride	BDH
Hydroquinone	BDH
Kanamycin	Sigma
Manganous chloride	BDH
Methhylene blue	Hopkin & Williams
Potassium acetate	BDH
Potassium chloride	BDH
tri-Potassium citrate	BDH
Potassium ferricyanide	BDH
Savalon (liquid)	ICI
Sodium chloride	BDH
Sodium hydroxide	BDH
Sodium thiosulphate	BDH
Silver nitrate	BDH
Sucrose	Fisons
TES	Sigma
Tetraethylammonium bromide	Sigma
Tetrodotoxin	Sigma
Trizma Base	Sigma
Verapamil hydrochloride	Abbot Laboratories Ltd

APPENDIX III DRUGS

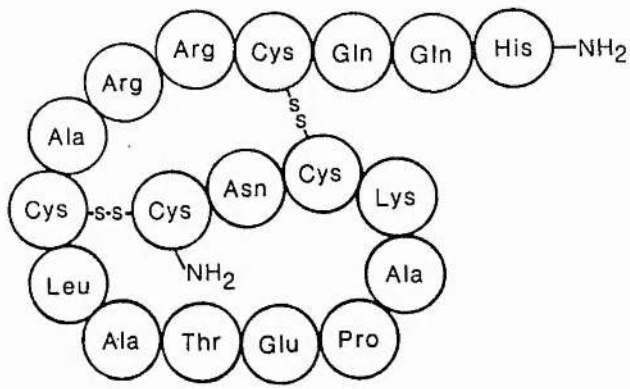
IIIB Drug structures.



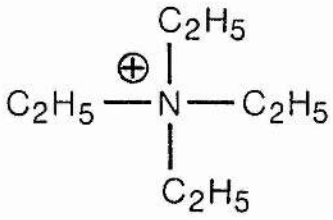
4-AP



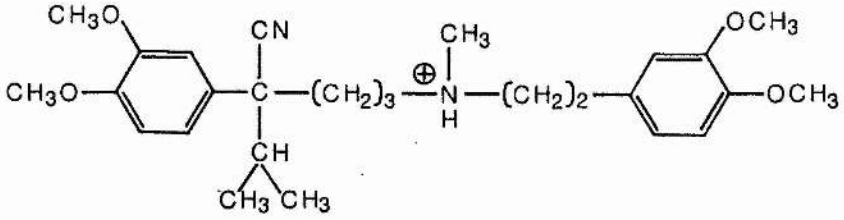
3,4-DAP



Apamin



TEA+



Verapamil

APPENDIX IV

PROTOCOL FOR PROCESSING PHOTOGRAPHIC FILM AND PAPER.

A. KODALITH ORTHO FILM TYPE 3.

PQ Universal (Ilford)	1+9	variable
Stop bath (KODAK)	1+36	10secs.
Hypam Fixative (ILFORD)	1+9	5mins.

Performed at 20°C under red safelight.

B. MULTIGRADE PAPER (II).

Multigrade developer (Ilford)	1+9	1min.
Stop bath	1+36	10secs.
Hypam Fixative	1+9	5mins.

Performed at 20°C under amber safelight.

C. RECORDAK AHU MICROFILM 5460.

D76 (KODAK)	1+2	12mins.
Wash in running water		2mins.
Hypam Fixative	1+9	variable.

Performed at 20°C in a developing tank.

APPENDIX V.

INTENSIFICATION PROCEDURE.

	Time (mins.)	Temperature (°C)
1. 1% Potassium ferricyanide in 1.25% sodium thiosulphate.	5	RT *1
2. Wash in 1.25% sodium thiosulphate in 50% ethanol.	10	RT
3. 50% ethanol	10	RT
4. 1.25% gum acacia in 30% ethanol	15	RT
5. 2.50% gum acacia in 30% ethanol	15	RT
6. 5.00% gum acacia in 30% ethanol	15	RT
7. 1.6% citric acid dissolved in 2% hydroquinone in solution 6.	60	RT
8. 0.1% silver nitrate in solution 7.	30-60	RT/DARK
9. 1.6% citric acid in solution 6.	10	4
10. 1.6% citric acid in solution 6.	10	4
11. 2.5% gum acacia in 30% ethanol.	15	45
12. 30% ethanol.	15	45
13. 50% ethanol.	15	RT
14. 70% ethanol.	variable	RT/4
15. 90% ethanol.	15	RT
16. 95% ethanol.	15	RT
17. 100% ethanol.	10	RT
18. 100% ethanol.	15	RT
19. Clear in histological creosote.	variable	RT/4
20. Wholemount the preparation on a cavity slide using canada balsam.		

*1 Where RT denotes room temperature.